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Method and Compositions Relating to Insulin ResistanceDisordersField of the Invention

5 The present invention relates to methods and compositions for the treatment of insulin resistance disorders including, but not limited to, non-insulin dependent diabetes. Specifically this invention identifies and describes proteins that are differentially expressed in 10 insulin resistance disordered states relative to their expression in normal or non-insulin resistance disordered states and also identifies proteins which are differentially expressed in response to manipulations relevant to alterations in insulin sensitivity. Further, 15 the present invention identifies and describes proteins by their ability to interact with proteins involved in the regulation of insulin resistance disorders and/or non-insulin dependent diabetes. Still further, the present invention provides methods for the identification 20 and therapeutic use of compounds or compositions as treatments for insulin resistance disorder including, but not limited to, non-insulin dependent diabetes. Additionally, the present invention describes methods for 25 the diagnostic evaluation and prognosis of various insulin resistant disorders, including predictive value of most appropriate therapy and for the identification of subjects exhibiting a predisposition to such conditions.

Background of the Invention

30 *Diabetes mellitus* is one of the most common metabolic disorders affecting more than 100 million people worldwide. Its global incidence is predicted to double by year 2010.

35 There are two types of diabetes. Type I, or insulin dependent diabetes mellitus (IDDM), is the result of

progressive autoimmune destruction of the pancreatic beta cells and constitutes 5-10% of the total diabetic population. Type II diabetes, also known as non-insulin dependent diabetes mellitus (NIDDM) or maturity onset diabetes, represents 90-95% of the diabetic population. Typically it occurs in middle-aged and elderly subjects, although it can develop in younger subjects and it is commonly associated with obesity. NIDDM is associated with two metabolic defects: insulin resistance and inappropriate insulin secretion. Insulin resistance affects particularly skeletal muscle and adipose tissues, resulting in reduced glucose uptake and also the liver, resulting in elevated hepatic production of glucose. Family studies indicate a major genetic component in the development of NIDDM, but apart from a sub-type of NIDDM called maturity onset diabetes of the young (MODY), few susceptibility genes have been identified.

Treatment for NIDDM has focused on the control of blood glucose. Current drugs, however, generally fail to achieve the same degree of control of blood glucose as is present in a non-diabetic subject. In addition, NIDDM patients often have elevated plasma triglycerides and elevated plasma cholesterol or a low ratio of HDL:LDL-cholesterol. All of these metabolic changes are adverse with respect to the development of secondary complications of diabetes, such as cardiovascular disease, blindness, nephropathy, stroke and microvascular disease.

Insulin resistance is a component of many other disorders besides NIDDM, including polycystic ovary syndrome, Cushing's disease, stroke, cardiovascular disease, hypertension, obesity and insulin dependent diabetes.

Animal models with mutations associated with insulin resistance disorders (including NIDDM) have been used as models for the study of the disorders. Among the best studied are mice, which contain the autosomal recessive mutations ob/ob (obese) and db/db (diabetes). These mutations are on chromosomes 6 and 4 respectively, but lead to clinically similar pictures, provided that the genes are expressed on the same background strain.

The ob gene product has been identified as a 16KDa polypeptide produced primarily by adipose tissue that provides a signal to the brain on the adipose tissue fat stores. Mice with a mutation, resulting in no circulating protein (called leptin) are hyperphagic, obese, have poor thermo-regulation and non-shivering thermogenesis and are insulin resistant with impaired glucose tolerance. When the ob gene mutation is on the C5B1/6 background, the mice do not present with manifest diabetes, but are massively hyperinsulinaemic.

The db/db mice have a mutation in the receptor for leptin so that normal signal transduction via the JAK/STAT pathway does not occur. This mutation, when on the C5B1/6 background, is phenotypically identical to the ob mutation. However, the db/db mutation is normally expressed on the C5B1/Ks mouse background and on this background the mutation causes frank diabetes. Other mouse mutations associated with insulin resistance, and which are models of non-insulin dependent diabetes, include the yellow mutation at the agouti locus, mutations at the fat and tubby loci and an autosomal dominant mutation at the adipose locus on chromosome 7.

Other mutant animal models include fa/fa (fatty) rats and ZDF fatty rats, which bear strong respective similarities

with the ob/ob and db/db mice. Thus the fa/fa rat is obese, insulin resistant, very hyperinsulinaemic and glucose intolerant, whereas the ZDF rat is obese, insulin resistant and hyperinsulinaemic, but develops frank diabetes after approximately 6 weeks of age.

5 Inbred mouse strains, such as the NZO mouse, the Japanese KK mouse and the GK rat are models of insulin resistance and diabetes. Further, desert rodents, such as spiny 10 mice and sand rats, are neither insulin resistant nor diabetic in their natural habitats, but do become insulin resistant and glucose intolerant when fed on a standard laboratory diet.

15 Insulin resistance and glucose intolerance are a common feature of elderly rodents and the development of insulin resistance in them can be accelerated by feeding diets with a high fat content, whether these diets are synthetic homogeneous diets or are the result of 20 supplementation or replacement of the normal rat chow by human food with a high fat content (cafeteria diet).

25 All of these animal models have been used from time to time to evaluate new drugs that are potential treatments for insulin resistance or non-insulin dependent diabetes in humans or companion animals such as dogs and cats. Largely this drug screening has been empirical and, although individual changes in some enzyme activities 30 have been identified in some of the animal models, there has been no systematic evaluation of the differences in protein expression in the tissues of insulin resistant animals in comparison with non-insulin resistant animals. It is these differences in protein expression that underlie the development of insulin resistance.

US Patent 5,702 902 (Tartaglia) identifies and describes genes which are differentially expressed at the mRNA level in body weight disorder states, relative to their expression in normal states. It is suggested therein that gene expression pattern can be used to identify compounds that can be used therapeutically to treat body weight disorders by either altering gene expression or by interacting with the gene products (proteins) of the differentially expressed genes.

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The use of differential gene expression as a tool for identifying the molecular basis of a disease process such as insulin resistance disorders relies on the differential gene mRNA expression being directly translated into a differential protein expression. This is not the case. The changes in protein expression is much more complex since the amount of protein present is influenced by the turnover rate of the corresponding mRNA, the turnover rate of the individual proteins, interaction of proteins with binding proteins and post-translational modification such as phosphorylation. Thus it is the changes in protein expression (including post-translational modification) that underlie the development of insulin resistance disorders including non-insulin dependent diabetes. It is these same changes in protein expression that are likely to be causative of insulin resistance disorders including non-insulin dependent diabetes in humans and companion animals such as cats and dogs.

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It has been a problem to find a more predictive method for the identification of the molecular basis of insulin resistance and thereby define the molecular targets that can be used to identify agents to treat the disease. It is also a problem to identify from the therapeutic tools

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available the most appropriate therapy for any individual with insulin resistance disorders given the severity and prevalence of insulin resistance disorders, particularly non-insulin dependent diabetes, there exists a great need for the systematic identification of the disease causing proteins, since modulation of the expression level of such proteins or the activity of such proteins in the subjects with insulin resistance disorders towards the level in normal or non-insulin resistant subjects represents a means of treating the condition.

Furthermore, since there are multiple causes of the overall insulin resistant state such methodologies will allow a prognosis to be made of the most appropriate and potentially most effective therapy to treat any individual suffering from the insulin resistant disorder.

**Summary of Invention**

It has now been found that the above problem is solved by a method based on the evaluation of the differential expression of proteins in tissues from subjects or animals having differential insulin sensitivity.

Broadly, the present invention relates to methods and compositions for the treatment of insulin resistance, including but not limited to, non-insulin dependent diabetes. Specifically the present invention identifies and describes proteins that are differentially expressed in insulin resistant states relative to their expression in normal, or non-insulin resistant states and also identifies proteins that are differentially expressed in response to manipulations relevant to insulin sensitivity regulation. Such differentially expressed proteins may represent 'target proteins' and/or fingerprint proteins. Further, the present invention identifies and describes proteins termed pathway proteins via their ability to

interact with proteins involved in the regulation of insulin sensitivity. Pathway proteins may also exhibit target protein and/or fingerprint protein characteristics.

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Accordingly, in a first aspect, the present invention provides a method of screening for an agent to determine its usefulness in treating insulin resistance, the method comprising:

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(a) establishing a paradigm in which at least one protein is differentially expressed in relevant tissue from, or representative of, subjects having differential levels of insulin sensitivity;

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(b) obtaining a sample of relevant tissue taken from, or representative of, an insulin resistant subject, who or which has been treated with the agent being screened;

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(c) determining the presence, absence or degree of expression of the differentially expressed protein or proteins in the tissue from, or representative of, the treated subject; and,

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(d) selecting or rejecting the agent according to the extent to which it changes the expression, activity or amount of the differentially expressed protein or proteins in the treated insulin resistant subject

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The paradigm may involve establishing at least one protein which is differentially expressed. However, in some embodiments, the paradigm may employ at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or 20 differentially expressed proteins.

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In a further aspect, the present invention provides a method for identification of an agent or agents for use in the treatment of insulin resistance disorders

comprising the steps of:

- (a) establishing of experimental and/or clinical paradigms that exhibit differential levels of insulin action, i.e. differential insulin sensitivity;
- 5 (b) identification of differentially expressed proteins in tissues of animals or humans exhibiting differential levels of insulin action; and,
- (c) selecting an agent that converts the expression and/or activity of one or more of the differentially expressed proteins in an insulin resistant state to the normal state for use in the treatment of the insulin resistance disorder state.

In a further aspect, the present invention provides a method of making a pharmaceutical composition which comprises having identified an agent using the above method, the further step of manufacturing the agent and formulating it with an acceptable carrier to provide the pharmaceutical composition.

20 In a further aspect, the present invention provides the use of an agent identified by the above method for the preparation of a medicament for the treatment of a condition characterised by insulin resistance.

25 In a further aspect, the present invention provides a method of treating a condition characterised by insulin resistance in a patient, the method comprising administering a therapeutically or prophylactically effective amount of such an agent identified by the above method.

30 In a further aspect, the present invention provides a method of determining the nature or degree of insulin resistance in a sample of relevant tissue from a human or

animal subject, which comprises:

(a) establishing a paradigm in which at least one protein is differentially expressed in relevant tissue from, or representative of, subjects having differential levels of insulin sensitivity,

(b) obtaining a sample of the tissue and

(c) determining the presence, absence or degree of expression of the differentially expressed protein or proteins in the sample, and

(d) relating the determination to the nature or degree of the insulin resistance by reference to a previous correlation between such a determination and clinical information.

Conveniently, the patient sample used in the method can be a tissue sample or body fluid sample or urine. This method allows the type of insulin resistance in a patient to be correlated to different types to prophylactic or therapeutic treatment available in the art, thereby enhancing the likely response of the patient to the therapy.

In a further aspect, the present invention provides a method of treatment by the use of an agent that will restore the expression of one or more differentially expressed proteins in the insulin resistant state to that found in the normal state in order to prevent the development of non-insulin dependent diabetes in a pre-diabetic subject.

In a further aspect, the present invention provides a method whereby the pattern of differentially expressed proteins in a tissue sample or body fluid sample or urine of an individual with insulin resistance is used to predict the most appropriate and effective therapy to

alleviate the insulin resistant state and to monitor the success of that treatment.

The present invention is based, in part, on systematic  
5 search strategies involving insulin resistance and non-  
insulin dependent diabetes experimental paradigms coupled  
with sensitive detection of differentially expressed  
proteins separated by physico-chemical methods. It has  
been found that two dimensional gel electrophoresis can  
10 be applied successfully to separate differentially  
expressed proteins implicated in insulin resistance  
disorders and in the treatment thereof. The invention  
therefore further provides differentially expressed  
proteins obtainable by this method, whether as spots on a  
15 gel or purified. However, as later described, other  
methods of producing differential expression of proteins  
are usable in the invention.

Accordingly, in a further aspect, the present invention  
20 provides a protein which is differentially expressed in  
relevant tissue from, or representative of subjects  
having differential levels of insulin sensitivity and  
which is obtainable by the method of two-dimensional gel  
electrophoresis carried out on said tissue or a protein-  
25 containing extract thereof, the method comprising:

- (a) providing non-linear immobilized pH gradient (IPG) strips of acrylamide polymer 3 mm x 180 mm;
- (b) rehydrating the IPG strips in a cassette containing 25 ml. of an aqueous solution of urea (8M), 3-[  
30 (cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS, 2% w/v), dithioerythritol (DTE, 10mM), mixture of acids and bases of pH 3.5 to 10 (2% w/v) and a trace of Bromophenol Blue;
- (c) emptying the cassette of liquid, transferring  
35 the strips to an electrophoretic tray fitted with humid

electrode wicks, electrodes and sample cups, covering the strips and cups with low viscosity paraffin oil;

(d) applying 200 micrograms of an aqueous solution of dried, powdered material of the relevant body tissue in urea (8M), CHAPS (4% w/v), Tris (40 mM), DTE (65 mM), SDS (0.05% w/v) and a trace of Bromophenol Blue to the sample cups, at the cathodic end of the IPG strips;

(e) carrying out isoelectric focusing on the gel at a voltage which increases linearly from 300 to 3500 V during 3 hours, followed by another 3 hours at 3500 V, and thereafter at 5000V for a time effective to enable the proteins to migrate in the strips to their pI-dependent final positions;

(f) equilibrating the strips within the tray with 100 ml of an aqueous solution containing Tris-HCl (50 mM) pH 6.8, urea (6M), glycerol (30% v/v), SDS (2% w/v) and DTE (2% w/v) for 12 minutes;

(g) replacing this solution by 100 ml. of an aqueous solution containing Tris-HCl (50 mM) pH 6.8, urea (6M), glycerol (30% v/v), SDS (2% w/v), iodoacetamide(2.5% w/v) and a trace of Bromophenol Blue for 5 minutes;

(h) providing a vertical gradient slab gel 160 x 200 x 1.5 mm of acrylamide/piperazine-diacrylyl cross-linker(9-16%T/2.6%C), polymerised in the presence of TEMED (0.5% w/v), ammonium persulphate (0.1% w/v) and sodium thiosulphate (5 mM), in Tris-HCl (0.375M) pH 8.8 as leading buffer;

(i) over-layering the gel with sec-butanol for about 2 hours, removing the overlay and replacing it with water;

(j) cutting the IPG gel strips to a size suitable for the second dimensional electrophoresis, removing 6 mm from the anode end and 14 mm from the cathode end;

(k) over-layering the slab gel with an aqueous

solution of agarose (0.5% w/v) and Tris-glycine-SDS (25 mM-198 mM-0.1% w/v) as leading buffer, heated to 70°C and loading the IPG gel strips onto the slab gel through this over-layered solution;

5 (l) running the second dimensional electrophoresis at a constant current of 40 mA at 8-12°C for 5 hours; and  
(m) washing the gel.

Examples of differentially expressed proteins include:

10 (a) proteins as obtainable from mouse liver cells of obese, insulin resistant mice, which may have been treated with rosiglitazone, and designated herein LOM16, LOM17, LOM18, LOMT19, LOM20, LOMT21, LOMT22, LOMT23, LOMT24, LOMT25, LOMT26, LOM27, LOM28, LOM29 or LSEM30;

15 (b) proteins as obtainable from skeletal muscle cells of obese, insulin resistant mice, which may have been treated with rosiglitazone, and designated herein MOM31, MOM32, MOM33, MOMT34, MOMT35 or MOM36;

20 (c) proteins as obtainable from white adipose tissue of obese, insulin resistant mice, which may have been treated with rosiglitazone, and designated herein WOMT37, WOM38, WOMT39, WOM40, WOM41, WOMT42, WOM43, WOM44, WOM45, WOM46, WOM47, WOMT48, WOMT49, WOMT50, WOM51 to 64 or WSEM65;

25 (d) proteins as obtainable from brown adipose tissue of obese, insulin resistant mice, which may have been treated with rosiglitazone, and designated herein BOM66, BOM67, BOMT68, BOM69 to 75, BOMT76 or BOM77.

30 In a further aspect, the present invention provides a method for screening for agents that are useful in treating insulin resistance which comprises:

35 (a) establishing a paradigm in which at least one protein is differentially expressed in a relevant tissue from, or representative of, subjects or animals having

differential levels of insulin sensitivity;

(b) identifying the differentially expressed protein;

5 (c) establishing a screen, preferably a high throughput in vitro screen, to detect compounds that will alter the expression level or the activity of the differentially expressed protein towards the level in the non-insulin resistant individual. Thus, the expression or activity should be increased if the protein is under-expressed in the resistant state and decreased if it is over-expressed;

10 (d) evaluating the effect of compounds that are regarded as positive effectors in (c) in animals or cell-based systems to determine if they modulate insulin sensitivity;

15 (e) evaluating the effect of positive effectors in (c) on the protein expression pattern of insulin resistant paradigms to determine if the protein expression pattern is changed towards that in the non-insulin resistant state;

20 (f) further selection of a therapeutic agent(s) based on conventional pharmaceutical parameters.

In a furter aspect the invention provides a method for 25 screening for agents that are useful in treating insulin resistance which comprises:

30 (a) establishing a paradigm in which at least one protein is differentially expressed in a relevant tissue from, or representative of, subjects or animals having differential levels of insulin sensitivity and in which the expression of one or more of the differentially expressed proteins are found to alter in a tissue from an insulin resistant animals or human by treatment with a known insulin sensitiser drug, or treatment such as 35 exercise that increases insulin resistance, towards the

protein expression pattern in the same tissue of animals or human which are non-insulin resistant or normal;

5 (b) identifying those proteins differentially expressed between the insulin resistant and normal state whose expression is altered favourably by treatment with a known insulin sensitiser drug or treatment such as exercise that increases insulin sensitivity;

10 (c) establishing a screen(s), preferably a high-throughput in vitro screen to detect further compounds that will alter the expression level or the activity of the proteins whose differential expression between insulin resistance or non-insulin resistant states was altered by the treatment of animals or humans exhibiting the insulin resistant state by insulin sensitiser drug or treatment. The objective of the in vitro screen is to identify compounds that produce similar changes in the differential protein expression pattern as that produced by the insulin sensitiser drugs or treatment. Such compounds identified by the in vitro screen are 15 potentially insulin sensitiser drugs but could be of a totally different nature to the insulin sensitiser drug or treatment used as the initial treatment insulin sensitiser drug;

20 (d) evaluating the effect of compounds that are regarded as positive effectors from (c) in animals or a cell-based system to determine if they modulate insulin 25 sensitivity.

30 (e) evaluating the effect of positive effectors from (c) on the protein expression pattern of insulin resistant paradigms to determine if the protein expression pattern is changed towards that in the non-insulin resistant state;

(f) further selection of a therapeutic agent(s) based on conventional pharmaceutical parameters.

In a further aspect the invention provides a method for screening for agents that are useful in treating insulin resistance with reduced side-effects which comprises:

(a) establishing a paradigm in which both insulin resistant and non-insulin resistant animals or humans are treated with an insulin sensitiser or drug and comparing the differential expression of proteins in tissues.

Proteins that are differentially expressed between the insulin resistant state and the non-insulin resistant state whose expression is altered favourably in the insulin resistant state but not in the non-insulin resistant state are potential molecular targets for agents to improve insulin sensitivity. Proteins that are either differentially expressed between animals or human with insulin resistant states or are not differentially expressed between insulin resistant states but whose expression is altered in either both insulin resistant states and non-insulin resistant states or just in the non-insulin resistant states by the treatment with an insulin sensitiser drug are potential molecular targets for the side-effects of that drug (side effect marker). Such side-effect marker proteins may be found in different tissues to the differentially expressed proteins associated with insulin resistance;

(b) identifying both those proteins that are targets for agents to improve insulin sensitivity and those proteins that are side-effect markers.

(c) establishing screens, preferably high-throughput in vitro screens to first detect further compounds that will favourable alter the expression level or the activity of the molecular target proteins and secondly to detect compounds that do not alter the expression or activity of the side-effect markers.

Compounds that are positive in the first but which have no effect in the second test are potential therapeutic

agents for insulin resistance disorders with reduced side-effects relative to the insulin sensitiser used in the original paradigm;

5 (d) evaluating the effect of compounds identified in (c) in animals or cell-based systems to determine if they modulate insulin sensitivity;

(e) evaluating the effect of compounds identified in (c) in animals or cell-based systems to determine if the side-effect profile is altered;

10 (f) evaluating the effect of such compounds from (c) on the protein expression pattern of insulin resistant paradigms and non-insulin resistant paradigms by administering the compounds to these animals and determining the changes in protein expression patterns;

15 (g) further selection of a therapeutic agent(s) based on conventional pharmaceutical parameters.

In another aspect the invention includes antibodies specific to the differentially expressed proteins especially monoclonal and recombinantly produced 20 antibodies and their use both as therapeutic agents and as diagnostic agents.

The invention includes the medical use of a target protein or an inhibitor thereof to alleviate an insulin 25 resistant state in a patient. In particular, it includes a method of treating an insulin resistant disorder in a patient, which comprises administering to the patient an amount of a target protein or inhibitor thereof, effective to alleviate the disorder.

30 **Definitions**

“Differential expression”, as used herein, refers to at least one recognisable difference in tissue protein expression. It may be a quantitatively measurable, semi-35 quantitatively estimatable or qualitatively detectable

difference in tissue protein expression. Thus, a differentially expressed protein (herein DEP) may be strongly expressed in tissue in the normal state and less strongly expressed or not expressed at all in tissue in the insulin resistant (disorder) state. Conversely, it may be strongly expressed in tissue in the disorder state and less strongly expressed or not expressed at all in the normal state. Similarly, the differential expression can be either way around in the comparison between untreated and treated tissue. Further, expression may be regarded as differential if the protein undergoes any recognisable change between the two states under comparison.

15 The term "paradigm" means a prototype example, test model or standard.

Wherever a differentially expressible protein is used in the screening procedure, it follows that there must have been at some time in the past a preliminary step of establishing a paradigm by which the differential expressibility of the protein was pre-determined. Once the paradigm has been established, it need not be re-established on every occasion that a screening procedure 25 is carried out. The term "establishing a paradigm" is to be construed accordingly.

"Insulin resistance" is a feature of conditions such as diabetes, especially NIDDM and its secondary 30 complications of diabetes, such as cardiovascular disease, blindness, nephropathy, stroke and microvascular disease, polycystic ovary syndrome, Cushing's disease, stroke, cardiovascular disease, hypertension and insulin dependent diabetes.

"Relevant tissue" means any tissue which undergoes a biological change in response to the action of insulin in the body, or any other tissue affected by this change.

5 "Tissue... ...representative of... ...subjects" means any tissue in which the above-mentioned biological change can be simulated for laboratory purposes and includes, for example, a primary cell culture or cell line derived ultimately from relevant tissue.

10 The term "subjects" includes human and animal subjects.

15 The treatments referred to above can comprise the administration of one or more drugs or foodstuffs, and/or other factors such as diet or exercise.

The differentially expressed proteins (DEPs) include "fingerprint proteins", "target proteins" or "pathway proteins".

20 The term "fingerprint protein", as used herein, means a DEP, the expression of which can be used, alone or together with other DEPs, to monitor or assess the condition of a patient suspected of suffering from 25 insulin resistance. Since these proteins will normally be used in combination, especially a combination of four or more, they are conveniently termed "fingerprint proteins", without prejudice to the possibility that on occasions they may be used singly or along with only one 30 or two other proteins for this purpose. Such a fingerprint protein or proteins can be used, for example, to diagnose a particular type of insulin resistance and thence to suggest a specific treatment for it.

35 The term "diagnosis", as used herein, includes the

provision of any information concerning the existence, non-existence or probability of the disorder in a patient. It further includes the provision of information concerning the type or classification of the disorder or of symptoms which are or may be experienced in connection with it. It encompasses prognosis of the medical course of the disorder.

10 The term "target protein", as used herein, means a DEP, the level or activity of which can be modulated by treatment to alleviate an insulin resistant disorder. Modulation of the level or activity of the target protein in a patient may be achieved, for example, by administering the target protein, another protein or gene 15 which interacts with it or an agent which counteracts or reduces it, for example an antibody to the protein, competitive inhibitor of the protein or an agent which acts in the process of transcription or translation of the corresponding gene.

20 The term "alleviate", as used herein, in relation to insulin resistance means any form of reducing one or more undesired symptoms or effects thereof. Any amelioration 25 of the insulin resistant condition of the patient falls within the term "alleviation".

30 Alternatively or additionally, the DEPs can interact with at least one other protein or with a gene involved in the regulation of insulin sensitivity. Such other proteins are termed herein "pathway proteins" (PPs). The term is applied to the protein with which it the DEP interacts, not to the DEP itself, although a pathway protein can be another DEP.)

35 The term "comprising", whenever used herein, means

"consisting of or including".

By way of example, embodiments of the present invention will now be described in more detail with reference to 5 the accompanying figures.

**Brief Description of the Figures**

Figures 1A-1E show computer images of stained 2-DGE gels 10 from liver cells of lean mice. Figure 1E shows the entire gel without identifying the spots thereon, while Figures 1A-1D are enlargements showing the four quadrants of the gel of Figure 1E and identifying spots thereon, including DEPs.

15 Figures 2-17 are computer mini-images of stained 2D-gels from liver cells of mice, showing differential expression of proteins (except in Figure 17, which is a control).

20 Figures 18A-18E, 24A-24E and 50A-50E show computer images of stained 2-DGE gels from skeletal muscle cells, white adipose tissue and brown adipose tissue, respectively, of mice. Commercial marker proteins which are available from Genomic Solutions have been used as references but these are not shown in the computer image pictures.

25 Figures 19-23, 25-49 and 51-62 show similar mini-images to those of Figures 2-16, except that the tissue is skeletal muscle cells, white adipose tissue and brown adipose tissue, respectively.

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**Detailed Description**

**Types of paradigm**

The invention uses differential expression of a protein as a means of identifying the locus of a change in 35 insulin sensitivity. The molecular mechanism of the

process by which insulin interacts with its receptor, resulting in signal transduction and the activation of a large number of metabolic events, including effects on glucose and lipid metabolism as well as effects on the transcription of many genes, is exceedingly complex and incompletely understood. Consequently, alterations in insulin could potentially involve changes in the concentration of a large number of proteins. Insulin resistance is also commonly associated with a large number of secondary complications that potentially could also alter the concentration of particular proteins in tissues. Thus, in trying to determine those proteins that are important to the regulation of insulin sensitivity, it is necessary to use defined experimental paradigms that will identify one or more of the proteins that are differentially expressed. It will be obvious to those skilled in the art that a single paradigm is unlikely to identify the molecular target for all causes of insulin resistance. Hence, it is an important judgement on the most appropriate paradigms to use to identify the differentially expressed proteins. A protein that is differentially expressed in many experimental paradigms of insulin resistance is potentially of major importance.

In a first embodiment of the paradigm, the invention makes use of those proteins that are differentially expressed between relatively insulin resistant and relatively insulin sensitive states. In principle, any two such states can be compared. Conveniently, tissues from normal and insulin resistant subjects are compared. Alternatively or additionally, the expression can be compared between normal and highly (abnormally) insulin sensitive individuals, such as those who regularly exercise.

This first type of paradigm yields DEP of group 1 type and is exemplified in the Figures by the designation "OM" (referring to an obese mouse model). It yields DEPs which are fingerprint proteins and may also be target proteins.

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For example, a DEP which is over-expressed in the normal state and under-expressed in the insulin resistant (disorder) state is a target for therapy to increase its production in the insulin resistant patient. Conversely, where the DEP is under-expressed in the normal state and over-expressed in the disorder state, it is a target for therapy to inhibit its production, expression or activity in the patient.

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15 In a second embodiment of the paradigm, an additional comparison of expression is introduced, namely between insulin resistant subjects and subjects who or which have been treated to increase their insulin sensitivity.

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These include treatment to alleviate insulin resistance by dietary restriction or modification, by exercise or by treatment with an insulin sensitising agent, typically a drug or a combination of drugs or by any combination of these parameters. A preferred such insulin-sensitising drug is one of the thiazolidinedione type, especially rosiglitazone, pioglitazone or troglitazone.

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30 The agent can be a non-thiazolidinedione, for example (a) an agonist or partial agonist of the PPAR gamma nuclear receptor, (b) a  $\beta_3$ -adrenoceptor agonist such as BRL 35135 or BRL 26830 or (c) leptin or leptin fragment. Other drugs include oxazolidinediones, such as JTT501, metformin and an RXR activator that forms a heterodimer with PPAR gamma.

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Such a paradigm will provide information about those proteins that are differentially expressed between insulin resistant and insulin sensitive states and whose expression is altered in the insulin resistant state by treatment of the insulin resistant subject with the particular insulin sensitising drug. Such DEPs are target proteins for the treatment of the patient.

This paradigm is of greater value for the detection of a target protein if the differential expression observed when the insulin resistant model subject has been treated with the drug is NOT also observed when an insulin-sensitive subject is likewise treated. Otherwise, the DEP might not genuinely reflect the insulin-sensitising action of the drug, but, rather, another action of the drug. Such other action can be regarded as a side-effect, although, of course, such a side effect is not necessarily noxious. Thus, stated a little more generally, in the third paradigm the protein or proteins differentially expressed in the treated insulin resistant subjects compared with normal subjects are differentially expressed in a reduced degree or not differentially expressed at all in the tissue of the treated normal subjects compared with the untreated normal subjects.

This third type of paradigm is exemplified in the Figures and yields a second group of DEPs designated "OMT" (referring to an obese mouse, treated model).

Paradigms in which a side-effect is manifested, through a change in differential expression being observed in an insulin-sensitive model subject as a result of treatment with a drug are also of value for two reasons. Firstly, the side effect may not be medically serious and/or may be manifested in an acceptably low degree. Secondly, such paradigms can be used to screen agents for side

effects. It constitutes a third group of DEPs designated "SEM" (referring to a side-effect marker) and is arrived at by detecting differential expression of a protein between untreated and treated subjects, both insulin-sensitive and insulin resistant subjects. Each such difference can independently be wider or over expression.

The essential difference between the first, second and third types of paradigm is that in the first the paradigm is not established by any treatment regime or drug, since normal subjects (e.g. lean control mice) are being compared with disorder subjects (e.g. obese mice), whereas in the second this comparison is supplemented by a further comparison between disorder subjects, untreated and treated (e.g. obese control mice and obese mice which have been treated with a drug). Thus, for example, when establishing paradigms, using lean and obese mice, with rosiglitazone as the drug for treatment, it is convenient to run four experimental groups at once, with lean control mice, obese control mice, lean treated mice and obese treated mice. The DEPs can then be grouped as follows:

Group 1 ("OM"): DEPs in the lean control v. obese control comparison, but which are NOT Group 2 DEPs.

Group 2 ("OMT"): DEPs in the lean control v. obese control comparison which are also DEPs in the obese treated v. obese control comparison, but which are NOT DEPs in a lean control v. lean treated comparison.

Group 3 ("SEM"): DEPs in the obese treated v. obese control comparison, but which are also DEPs in the lean treated v. lean control comparison.

It will be appreciated that each such type 1, type 2 or type 3 paradigm as illustrated above relates to a particular treatment, in this case with rosiglitazone. When another insulin-sensitising drug "X" (known or yet to be discovered) is used to establish a paradigm, it can be expected that there will be some differences. For example, some DEPs which are in Group 1, being "rosiglitazone-insensitive", may additionally be sensitive to drug "X" and therefore appear in Group 2 in the new paradigm. If, further, the lean treated v. lean control comparison of this DEP shows no major difference, it will be placed in Group 2.

Model subjects for the paradigm

Conveniently the paradigm is established "experimentally" in animal models, for example rodents, although human or animal subjects can be used to provide a "clinical" paradigm.

In one embodiment, the comparison is designed to detect DEPs involved in genetic or heritable insulin resistant disorders, including NIDDM. In the case of mice, for example, such a comparison may identify the proteins regulated either directly or indirectly by the ob/ob, tub or fat gene products. In rats it may identify proteins regulated either directly or indirectly by the fa gene product. In one particular paradigm, the subjects may include ob/ob, db/db, tub/tub or fat/fat experimental mice and lean littermate controls or fa/fa or 2DF rats and lean controls. Such animals can be offered a normal chow diet for a given period, after which tissue samples are collected for analysis.

In additional embodiments, ob/ob, db/db, tub/tub and/or fat/fat mice and/or fa/fa rats and lean control animals

may be treated with drugs that improve insulin sensitivity. Such drugs include insulin sensitizers as described above.

5 In a further type of comparison, ob/ob, db/db, tub/tub and/or fat/fat mice and/or fa/fa rats and/or non-mutant animals may be offered dietary treatments to either worsen the insulin resistant state or improve insulin sensitivity. For example, either insulin sensitive or 10 insulin resistant animals could be provided with a high fat diet to exacerbate the insulin resistant state. In one embodiment thereof, young and old mice or rats are fed on a high fat diet or a cafeteria diet consisting of human snack foods. Young rats weighing less than 200g 15 resist the development of insulin resistance when fed on a high fat or cafeteria diet, whereas rats weighing 350g or more rapidly become insulin resistant. Thus, this method can be used to detect DEPs that are associated with the insulin resistant state. It can be further 20 refined by incorporating comparisons based on drug treatment, as described above.

Some native animal strains do not exhibit either insulin resistance or non-insulin dependent diabetes in the wild 25 but do when fed a laboratory chow or other laboratory diets. These include the desert rodents, the spiny mouse and the sand rat. Comparison of animals fed on the natural diet and those fed on a laboratory diet allows the detection of DEPs associated with insulin resistance.

30 In another embodiment to ascertain DEPs, pregnant mice or rats may be fed on a low protein diet (typically 6-8% protein) during pregnancy and/or lactation whereas control animals are fed on a diet containing a normal 35 protein content (typically 15%). After weaning,

offspring may be fed on a high fat diet. Such latter offspring develop insulin resistance and non-insulin dependent diabetes. Comparison between these various animals fed on different diets during gestation, weaning and/or in adult life allows the detection of DEPs associated with insulin resistance.

The paradigm can be established clinically using subjects with, for example, (a) non-insulin dependent diabetes, impaired glucose tolerance, obesity, polycystic ovary syndrome, Cushing syndrome, Syndrome X, or insulin resistance syndrome as representative of the disorder state, (b) normal individuals and (c) individuals who have exercised regularly as representative of the abnormally insulin sensitive state, using any two or all of (a), (b) and (c).

The model subject may be a transgenic or knock-out animal (usually a mouse), which has an alteration in its insulin sensitivity relative to the wild-type as a result of either overexpressing and/or knock out of one or more genes.

**Types of tissue used in establishing the paradigm**

The paradigm can be established using any cellular tissue susceptible to the action of insulin and thus capable of undergoing changes in insulin sensitivity. These include, for example, skeletal muscle(s), liver and brown and white adipose tissue(s). Whole cellular tissue or a sub-cellular fraction or extract thereof may be used. Particularly useful is a nuclear protein fraction.

**Cell models for the Paradigm**

The paradigm can also be established in cultured cells or in a cell line which is representative of in vivo body

tissues.

The cells could be primary cultures of cells, for example adipocytes, obtained from any of the embodiments listed 5 earlier. Alternatively the cells could be a cell-line such as the pre-adipocyte cell lines such as 3T3-L1 or F42A mouse pre-adipocyte cell-line, liver cell-lines such as the Hepal-6 mouse liver cell-line and the HepG2 human liver cell-line or muscle cell lines such as the L6 or 10 C<sub>2</sub>C<sub>12</sub> cell lines.

In one particular embodiment we envisage samples of a 15 cell-line in a microtitre plate format being treated with a range of drugs that are known to have potential to modulate insulin sensitivity and the DEPs being identified.

#### Methods of detecting differential expression

The preferred method of detecting differential expression 20 in the paradigm is two-dimensional gel electrophoresis (2-DGE). In 2-DGE, a sample containing a mixture of proteins is subjected to isoelectric focusing in one dimension, to separate proteins according to their charge and to electrophoresis in another dimension, to separate 25 proteins according to their relative molecular mass (molecular weight). Second dimension electrophoresis is normally by SDS-PAGE. After staining, a complex pattern of spots is obtained. This may be likened to a two-dimensional map. When the sample is taken from a patient 30 suffering from an abnormality, the success of the technique depends on comparing the map resulting from an abnormal sample with that resulting from a normal sample and looking for differences. Thus, whether the technique 35 will work in relation to any particular abnormality depends on many complex factors, including whether any

spots can be detected which are present in the abnormal tissue but not the normal or present in the normal tissue but not the abnormal. When spots are present in abnormal and normal tissue, but at different densities,

5 representing different concentrations in the tissue, the analysis becomes difficult. When there are differences in the expression of proteins, these might be hidden by other spots, common to both kinds of tissue or might not be reliably observable. In the present invention,  
10 however, it has been found possible by 2-DGE to identify spots corresponding to DEPs implicated in insulin resistance and its treatment.

15 Other methods of detecting differential expression of proteins can be used. These include successive chromatographic separations of fractions and comparisons of the peaks, capillary electrophoresis and separations using micro-channel networks, including on a micro-chip.

20 Chromatographic separations can be carried out by high performance liquid chromatography as described in Pharmacia literature, the chromatogram being obtained in the form of a plot of absorbance of light at 280 nm against time of separation. The material giving  
25 incompletely resolved peaks is then re-chromatographed and so on.

30 Capillary electrophoresis is a technique described in many publications, for example in the literature "Total CE Solutions" supplied by Beckman with their P/ACE 5000 system. The technique depends on applying an electric potential across the sample contained in a small capillary tube. The tube has a charged surface, such as negatively charged silicate glass. Oppositely charged  
35 ions (in this instance, positive ions) are attracted to

the surface and then migrate to the appropriate electrode of the same polarity as the surface (in this instance, the cathode). In this electroosmotic flow (EOF) of the sample, the positive ions move fastest, followed by uncharged material and negatively charged ions. Thus, proteins are separated essentially according to charge on them.

Micro-channel networks function somewhat like capillaries and can be formed by photoablation of a polymeric material. In this technique, a UV laser is used to generate high energy light pulses that are fired in bursts onto polymers having suitable UV absorption characteristics, for example polyethylene terephthalate or polycarbonate. The incident photons break chemical bonds with a confined space, leading to a rise in internal pressure, mini-explosions and ejection of the ablated material, leaving behind voids which form micro-channels. The micro-channel material achieves a separation based on EOF, as for capillary electrophoresis. It is adaptable to micro-chip form, each chip having its own sample injector, separation column and electrochemical detector: see J.S. Rossier et al., 1999, Electrophoresis 20: pages 727-731.

#### Conduct of two-dimensional gel electrophoresis

2-DGE is preferably carried out on acrylamide polymer gels and is then known as 2-D-PAGE.

Normally, the first dimensional separation comprises isoelectric focusing (IEF), an electrophoretic technique in which amphoteric compounds are fractionated according to their isoelectric points (pIs) along a substantially continuous pH gradient. In the process of IEF with carrier ampholytes, a pH gradient, increasing from anode

to cathode, is created and maintained, by the passage of an electric current through a solution of amphoteric compounds which have closely spaced pIs, encompassing a given pH range. Their electrophoretic transport causes 5 the carrier ampholytes to stack according to their pIs. After this stacking process is completed, some carrier ampholytes still enter zones of higher or lower pH, by diffusion, where they are no longer in isoelectric equilibrium. However, as soon as they enter these zones, 10 they become charged and the applied voltage forces them back to their equilibrium position, and so on in a kind of pendulum movement.

15 In the preferred process of IEF with immobilized pH gradient (IPG), gel strips are prepared to have a given pH range, which exists prior to the IEF run itself. Effectively, carrier ampholytes are replaced by various monomers which are copolymerised with acrylamide, and thus provide insolubilised ions within the polyacrylamide 20 matrix. These monomers are a set of weak acids and bases, preferably those sold under the Registered Trade Mark "Immobiline". When the field is applied, only the sample molecules (and any residual ions which were not 25 insolubilised) migrate in the electric field. Upon termination of electrophoresis the proteins are separated into stationary isoelectric zones.

30 In the second dimension separation of proteins in polyacrylamide gel, buffers are used which dissociate proteins into their individual polypeptide subunits. The most common dissociating agent used is the ionic detergent, sodium dodecyl sulphate (SDS). The protein mixture is denatured in the presence of excess SDS and a 35 thiol reagent to cleave disulphide bonds. Under these conditions, most polypeptides bind SDS in a constant

weight ratio (about 1.4 g of SDS per gram of polypeptide). The intrinsic charges of the polypeptide are insignificant compared to the large negative charges provided by the bound detergent, so that the SDS-  
5 polypeptide complexes have essentially identical charge densities and migrate in polyacrylamide gels of the correct porosity strictly according to polypeptide size and therefore relative molecular mass.

10 The 2-DGE spots can be visualised by staining, optionally followed by photography. However, more accurate representations are available by densitometric scanning, followed by computer imaging of the results.  
In this way sophisticated databases can be built up.

15

#### Definition of the DEPs

The DEPs can be defined by the position of their spots on a 2-DGE map, in terms of the position of the spots relative to other protein spots. Alternatively, although 20 less desirably, they can be defined in terms of co-ordinates of pI, usually the abscissa (x-axis) and relative molecular mass, usually the ordinate (y-axis). Although pI is reasonably reproducible when the IPG strips are used, the relative molecular mass is less 25 reproducible from one run to another. For this reason, the drawings show "reference" spots, corresponding to proteins which are not differentially expressed under the same defined, comparable conditions as the spots corresponding to DEPs. At least one, usually two or 30 more, of these reference spots will serve to indicate the position of the DEP spots.

35 After staining and desitometric scanning of the spots, their relative densities can be estimated by computer programs in terms of the percentage volume or optical

density of a particular spot, expressed in terms of the total volume or optical density, respectively, of all spots on the map, although these measurements might vary from run to run.

5 The preferred gel electrophoretic conditions, under which the maps shown in the drawings were produced, use a sigmoidal IPG for isoelectric focusing and a conventional SDS polyacrylamide gel with some small 10 modifications (addition of piperazine-diacrylyl as crosslinker and sodium thiosulphate to reduce background in the staining of the gel) in the second dimension. A sensitive ammoniacal silver stain was used. After 15 scanning, the 2-D-PAGE pictures were analysed with the "Melanie II" image analysis software.

20 The DEPs may conveniently be defined as the proteins of the differentially expressed spots obtainable by the 2-DGE method actually used. In more detail this method comprises:

25 (a) providing non-linear immobilized pH gradient (IPG) strips of acrylamide polymer 3 mm x 180 mm;  
(b) rehydrating the IPG strips in a cassette containing 25 ml. of an aqueous solution of urea (8M), 3-[  
26 (cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS, 2% w/v), dithioerythritol (DTE, 10mM), mixture of acids and bases of pH 3.5 to 10 (2% w/v) and a trace of Bromophenol Blue;  
30 (c) emptying the cassette of liquid, transferring the strips to an electrophoretic tray fitted with humid electrode wicks, electrodes and sample cups, covering the strips and cups with low viscosity paraffin oil;  
(d) applying approximately 200 micrograms of an aqueous solution of dried, powdered material of the relevant body tissue in urea (8M), CHAPS (4% w/v), Tris (40 mM), DTE  
35

(65 mM), SDS (0.05% w/v) and a trace of Bromophenol Blue to the sample cups, at the cathodic end of the IPG strips;

5 (e) carrying out isoelectric focusing on the gel at a voltage which increases linearly from 300 to 3500 V during 3 hours, followed by another 3 hours at 3500 V, and thereafter at 5000V for a time effective to enable the proteins to migrate in the strips to their pI-dependent final positions;

10 (f) equilibrating the strips within the tray with 100 ml of an aqueous solution containing Tris-HCl (50 mM) pH 6.8, urea (6M), glycerol (30% v/v), SDS (2% w/v) and DTE (2% w/v) for 12 minutes;

15 (g) replacing this solution by 100 ml. of an aqueous solution containing Tris-HCl (50 mM) pH 6.8, urea (6M), glycerol (30% v/v), SDS (2% w/v), iodoacetamide(2.5% w/v) and a trace of Bromophenol Blue for 5 minutes;

20 (h) providing a vertical gradient slab gel 160 x 200 x 1.5 mm of acrylamide/piperazine-diacyryl cross-linker(9-16%T/2.6%C), polymerised in the presence of TEMED (0.5% w/v), ammonium persulphate (0.1% w/v) and sodium thiosulphate (5 mM), in Tris-HCl (0.375M) pH 8.8 as leading buffer;

25 (i) over-layering the gel with sec-butanol for about 2 hours, removing the overlay and replacing it with water;

(j) cutting the IPG gel strips to a size suitable for the second dimensional electrophoresis, removing 6 mm from the anode end and 14 mm from the cathode end;

30 (k) over-layering the slab gel with an aqueous solution of agarose (0.5% w/v) and Tris-glycine-SDS (25 mM-198 mM-0.1% w/v) as leading buffer, heated to 70°C and loading the IPG gel strips onto the slab gel through this over-layered solution;

35 (l) running the second dimensional electrophoresis at a constant current of 40 mA at 8-12°C for 5 hours; and

(m) washing the gel.

Specific such DEPs of the invention are those hereinafter identified in the Figures by "OM", "OMT" and "SEM" 5 designations, preceded by a letter denoting the tissue (L = liver, M = skeletal muscle, W = White adipose and B = Brown adipose). The invention includes such DEPs *per se*, as obtainable by (but not necessarily actually obtained by) the above-recited 2-DGE method. It will be 10 appreciated that once the DEPs have been obtained by 2-DGE, they can be identified and synthesised, e.g. by a recombinant DNA route.

**Use of the DEPs**

15 All the DEPs obtained in this invention are diagnostically useful. Those of Group 1 are fingerprint proteins and may be target proteins, those of Group 2 are target proteins and those of Group 3 are useful in indicating a potential or actual side-effect of the test 20 agent being screened. Further, all of them are useful for the purpose of raising antibodies against them, in order to enable expression of the DEPs in the screening to be determined by immunoassay using these antibodies.

25 **Identification and characterisation of the DEPs**

Firstly, the protein spots can be excised from the gel and partially amino acid sequenced by conventional methods. They can also be analysed by peptide mass 30 fingerprinting using mass spectrometric techniques well known in the art. In this technique, the very accurately determined masses of peptide fragments are matched to mass-fragment databases to give probable sequences. Where the protein is suspected to correspond to one which 35 is already known and against which an antibody is available, its identity can be confirmed by

immunoblotting.

Secondly, the DEPs can be characterised by obtaining a partial amino acid sequence (as above) and then 5 identifying the corresponding gene. This can be done by constructing a pool of oligonucleotide probes or PCR primers, which are used to screen cDNA libraries of the relevant tissue (preferably that from which the DEP was obtained by 2-DGE). Using hybridisation or PCR 10 techniques well known in the art, the DNA in the tissue coding for the protein can then be identified. The protein predicted by the relevant open-reading frame of the DNA is then identified as the DEP.

15 Thirdly, an analysis of the tissue and/or cell type distribution of the protein may be made directly on the distribution of the corresponding mRNA, using standard techniques well known in the art. They include, for example, Northern, RNase protection and RT-PCR analyses.

20 Such analyses provide information as to, for example, whether the identified proteins are expressed in tissues or cell types expected to contribute to the insulin resistance disorder of interest. Such an analysis may 25 provide information regarding the biological function of an identified protein in relation to the insulin resistance disorder in instances wherein only a subset of the cells within the tissue is thought to be relevant to the insulin resistance disorder.

30 Fourthly, the biological function of the identified proteins may be more directly assessed *in vivo* or *in vitro*. Thus, animals which (a) naturally exhibit insulin 35 resistant disorder-like symptoms, (b) have been induced to exhibit such symptoms or (c) which are transgenic or "knock out" can be used. Such animals are further

exemplified below. Cells of a type known or suspected to contribute to the insulin resistant disorder of interest can be used. They may be wild type cells, or may be non-wild type cells containing modifications known to, or suspected of, contributing to the insulin resistant disorder of interest. Such systems are discussed in detail below.

In further characterising the biological function of the DEPs, the expression of these proteins may be modulated within the *in vivo* and/or *in vitro* systems, i.e. either over-expressed or under-expressed in, for example, transgenic animals and/or cell lines, and its subsequent effect on the system then assayed. Alternatively, the activity of the identified protein may be modulated by either increasing or decreasing the level of activity in the *in vivo* and/or *in vitro* system of interest, and its subsequent effect then assayed.

The information obtained through such characterisations may suggest relevant methods for the treatment of insulin resistant disorders involving the protein of interest. Further, relevant methods for the control of non-insulin dependent diabetes involving the protein of interest may be suggested by information obtained from such characterisations. For example, treatment may include a modulation of protein expression and/or protein activity. Characterisation procedures such as those described herein may indicate where such modulation should involve an increase or a decrease in the expression or activity of the protein of interest. Such methods of treatment are discussed below.

**Detection, identification and characterization of pathway proteins**

Methods are described herein for the detection and identification of pathway proteins (PPs), which interact with DEPs involved in insulin resistant disorders. A PP may itself be differentially expressed. It may have the characteristics of a target protein or be useful 5 diagnostically as a fingerprint protein.

Any method suitable for detecting protein-protein interactions may be employed for identifying PPs by 10 identifying interactions between unknown proteins (candidate PPs) and DEPs. Such candidate PPs may be cellular or extracellular proteins.

Among the traditional methods, which may be employed, are 15 co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns. Using procedures such as these allows for the detection of PPs. Once detected, a PP may be used, in conjunction with standard techniques, described above in connection with 20 characterisation of DEPs, to identify its corresponding pathway gene.

One method, which detects protein interactions *in vivo*, the two-hybrid system, has been described (Chien *et al.* 25 1991, Proc. Natl. Acad. Sci. USA, 88, 9578-9582) and is commercially available from Clontech (Palo Alto, Calif.). Briefly, plasmids are constructed that encode two fusion 30 proteins. The first plasmid contains DNA encoding a fusion protein of the DNA-binding domain of a transcription activator protein, e.g. GAL4, fused to a known protein, in this case, the DEP. The other plasmid contains DNA encoding a fusion protein of the activator domain of the same transcription activator protein, fused to the unknown protein (the candidate PP). The unknown 35 protein is encoded by a cDNA which has been recombined

into this plasmid as part of a cDNA library. The two plasmids are transformed into a strain of the yeast *Saccharomyces cerevisiae* that contains a reporter gene, e.g. *lacZ*, whose regulatory region contains the transcription activator's binding sites. Either hybrid protein alone cannot activate transcription of the reporter gene: the DNA-binding domain hybrid cannot because it does not provide activation function and the activation domain hybrid cannot because it cannot localise to the activator protein's binding sites.

Interaction of the two hybrid proteins reconstitutes the functional transcriptional activator protein, exemplified as GAL4, and results in expression of the reporter gene, e.g. *lacZ*. This is detected by an assay for the reporter gene product, which in the example is  $\beta$ -galactosidase, giving a blue colour on Xgal plates.

Colonies of the transformed *S. cerevisiae* expressing the reporter gene are purified and the library plasmids responsible for reporter gene expression are isolated. DNA sequencing is then used to identify the pathway protein encoded by the library cDNA.

The cDNA of the unknown protein can then be purified from these strains, and used to produce and isolate the pathway protein using techniques routinely practised in the art.

Protein interactions can also be monitored and analysed using the Biocore™ system for monitoring biomolecular binding. Biocore™ technology enables direct detection and monitoring of biomolecular binding events for rapid assessment of method development and purification of these biomolecules. A target biomolecule, such as a differentially expressed protein, is attached to the

surface of a sensor and aliquots of the sample passed over this surface. When a further protein binds to the primary protein on the sensor surface (a hit) there is a change in mass concentration close to the surface. This 5 change in concentration is detected in real time, providing the opportunity to monitor the binding of native protein from complex mixtures to a target protein without prior introduction of labels or tags. The bound protein is then removed from the surface of the sensor 10 chip, and purified by conventional methods (Nordhoff et al, Nat. Biotech. 17(9):884-888, 1999). Biocore™ technology can provide information about the kinetics, affinity and specificity of protein interactions. Thus 15 the Biocore™ technology can allow the detection of pathway proteins.

Once identified pathway proteins can be further characterized in the same manner as differentially expressed target proteins.

20

#### Antibodies specific for DEPs or PPs

This section describes methods for the production of 25 antibodies capable of specifically recognising one or more epitopes of a DEP (which may be a target protein) or PP on a fingerprint protein. Such antibodies may include polyclonal antibodies, monoclonal antibodies (mAbs), humanised or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')<sub>2</sub> fragments, fragments produced by a FAb expression library, anti-idiotypic 30 (anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may be used as part of insulin resistance treatment methods, and/or may be used as part of diagnostic techniques whereby patients may be tested for abnormal levels or forms of DEP or PP.

35

For the production of antibodies to DEP or PP, various host animals may be immunised by injection with a DEP or PP, or a peptide thereof. The peptide may be conjugated to a carrier, e.g. KLH, in a manner well known in the art. Such host animals include rabbits, mice and rats. Various adjuvants may be used to increase the immunological response, depending on the host species, including active substances such as lysolecithin, polyoxyethylene polyols, polyanions, peptides, oil emulsions, and potentially useful human adjuvants such as BCG and *Corynebacterium parvum*.

10 Polyclonal antibodies are obtainable from the sera of animals immunised with the DEP or PP, or an antigenic 15 functional derivative thereof.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique, which provides for the production of 20 antibody molecules by continuous cell lines in culture. These include the hybridoma technique of Köhler and Milstein, 1975, *Nature* 256, 495-497; and US Pat. No. 4,376,110, the human B-cell hybridoma technique (Kosbor et al., 1983, *Immunology Today* 4: 72; Cole et al., 1983, 25 Proc. Natl. Acad. Sci. USA 80; 2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, "Monoclonal Antibodies and Cancer Therapy", Alan R. Liss Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass 30 thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or, preferably, *in vivo*.

In addition, techniques developed for the production of 35 chimeric antibodies (Morrison et al., 1984, Proc. Natl.

Acad. Sci. 81: 6851-6855; Neuberger *et al.*, 1984, Nature 312: 604-608; Takeda *et al.*, 1985, Nature 314: 452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (US Pat. No. 4,946,778; Bird, 1988, Science 242: 423-426; Huston *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85: 5879-5883; and Ward *et al.*, 1989, Nature 334: 544-546) can be adapted to produce DEP or PP single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments, which recognise specific epitopes, may be generated by known techniques. For example, such fragments include the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternative, Fab expression libraries may be constructed (Huse *et al.*, 1989, Science 246: 1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

**Screening for agents to ameliorate insulin resistance**

Any of the differentially expressed proteins whose

5 further characterisation indicates that a modulation of the expression of the protein or a modulation of the activity of the protein may ameliorate any of the insulin resistant disorders is designated a target protein. Such target proteins, along with those discussed below, will constitute the focus of compound discovery strategies as discussed. Such target proteins and/or modulating compounds can be used as part of the treatment of insulin resistance disorders including, but not limited to, non-  
10 insulin dependent diabetes.

15 Furthermore, pathway proteins which may subsequently be found to be differentially expressed and whose further characterisation indicates that modulation of the expression of the protein or a modulation of the activity of the protein may ameliorate any of the insulin resistant disorders, will also be designated a target protein and will constitute the focus of compound discovery strategies.

20 In addition, one or more pathway proteins may reveal a lack of differential expression but characterisation may indicate that modulation of the expression or activity of the proteins may nonetheless ameliorate insulin  
25 resistance disorders. In such cases, these pathway proteins would also be considered a focus of the compound discovery strategies.

30 The characterisation of target proteins may suggest relevant methods for the treatment of insulin resistance disorders including the treatment of non-insulin dependent diabetes. For example, the characterisation procedures may indicate where such modulation should involve an increase or a decrease in the expression or  
35 activity of the target protein(s) of interest.

A key component of the compound discovery strategy is to develop a screen, preferably an in vitro high-throughput or ultra-high throughput screen, although any screen could be used that provided the ability to detect 5 compounds that acted on the target protein(s) or modulated the expression level of target protein(s) in a manner consistent with favourable modulation of insulin sensitivity. The screen could be a subcellular assay or cell-based or whole animal based.

10

In one embodiment of the invention the screen would be designed to determine the effect of compounds on the expression level of the target protein. Techniques involving the obtention of the recombinant target gene, 15 together with regulatory sequences are well described, as are methodologies for establishing a reporter gene assay using the regulatory sequences of the target gene. Such assays could be used to identify compounds that modulated the expression level of the target protein in a direction 20 consistent with an increase in insulin sensitivity.

In a further embodiment screening assays for compounds that interact with the target protein would be utilised.

25 Thus, transgenic animals, in addition to providing proof that the target protein(s) is causative in the development of insulin resistance can also be used to screen the compounds that might ameliorate the insulin resistance by altering the expression or modifying the 30 activity of the target protein expressed as a transgene. Such animals have a particular value when the transgene is the human target protein.

35 The following assays are designed to identify compounds that bind to target proteins, bind to other cellular

proteins that interact with a target proteins, and to compounds that interfere with the interaction of the target proteins with other cellular proteins. Such compounds may include, but are not limited to, other cellular proteins. Methods for the identification of such cellular proteins are described below.

Compounds may include, but are not limited to, peptides such as, for example, soluble peptides, including, but not limited to, Ig-tailed fusion peptides, comprising extracellular portions of target protein transmembrane receptors, and members of random peptide libraries (see, e.g. Lam, K.S. et al., 1991, *Nature* 354: 82-84; Houghton, R. et al., 1991, *Nature* 354: 84-86) made of D- and/or L-configuration amino acids, phosphopeptides (including, but not limited to, member of random or partially degenerate, directed phosphopeptide libraries: see, e.g. Songyang, Z. et al., 1993, *Cell* 72: 767-778), antibodies (including, but not limited to, polyclonal, monoclonal, humanised, anti-idiotypic, chimeric or single chain antibodies, and FAb, F(ab')<sub>2</sub> and FAb expression library fragments, and epitope-binding fragments thereof) and small organic or inorganic molecules.

Compounds identified via assays such as those described herein may be useful, for example, in elaborating the biological function of the target protein, and for ameliorating insulin resistance disorders. In instances, for example, whereby an insulin resistance disorder situation results from a lower overall level of target protein expression and/or target protein activity in a cell or tissue involved in such an insulin resistance disorder, compounds that interact with the target protein may include ones which accentuate or amplify the activity of the bound target protein. Such compounds would bring

about an effective increase in the level of target protein activity, thus ameliorating symptoms. In instances whereby mutations within the target gene cause aberrant target proteins to be made which have a deleterious effect that leads to an insulin resistance disorder, compounds that bind target protein may be identified that inhibit the activity of the bound target protein.

10 In vitro screening for compounds that bind to the target proteins

In vitro systems may be designed to identify compounds capable of binding the target proteins of the invention. Compounds identified may be useful, for example, in 15 modulating the activity of wild type and/or mutant target proteins, may be useful in elaborating the biological function of the target protein, may be utilised in screens for identifying compounds that disrupt normal target protein interactions, or may in themselves 20 disrupt such interactions.

The principle of the assays used to identify compounds that bind to the target protein involves preparing a reaction mixture of the target protein and the test 25 compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected in the reaction mixture. These assays can be conducted in a variety of ways. For example, one method to conduct such 30 an assay would involve anchoring target protein or the test substance onto a solid phase and detecting target protein/test compounds complexes anchored on the solid phase at the end of the reaction. In one embodiment of such a method, the target protein may be anchored onto a 35 solid surface, and the test compound, which is not

anchored, may be labelled, either directly or indirectly.

In practice, microtiter plates may conveniently be utilised as the solid phase. The anchored component may 5 be immobilised by non-covalent or covalent attachments.

Non-covalent attachment may be accomplished by simply coating the solid surface with a solution of the protein and drying. Alternatively, an immobilised antibody, preferably a monoclonal antibody, specific for the 10 protein to be immobilised may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the non-immobilised 15 component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (e.g. by washing) under conditions such that any complexes formed will remain immobilised on the solid surface. The detection of 20 complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously non-immobilised component is pre-labelled, the detection of label immobilised on the surface indicates that complexes were formed. Where the previously non- 25 immobilised component is not pre-labelled, an indirect label can be used to detect complexes anchored on the surface, e.g. using a labelled antibody specific for the previously non-immobilised component (the antibody, in turn, may be directly labelled or indirectly labelled 30 with a labelled anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected, e.g. using an 35 immobilised antibody specific for target protein or the

test compound to anchor any complexes formed in solution, and a labelled antibody specific for the other component of the possible complex to detect anchored complexes.

5       Assays for cellular proteins that interact with the target protein

Any method suitable for detecting protein-protein interactions may be employed for identifying novel target protein-cellular or extracellular protein interactions. 10 These methods are outlined above, for the identification of pathway proteins, and may be utilised herein with respect to the identification of proteins which interact with identified target proteins.

15       Assays for compounds that interfere with target protein/cellular macromolecule interaction

The target proteins of the invention may, *in vivo*, interact with one or more cellular or extracellular macromolecules, such as proteins. Such macromolecules 20 may include, but are not limited to, nucleic acid molecules and those proteins identified via methods such as those described above. For purposes of this discussion, such cellular and extracellular macromolecules are referred to herein as 'binding 25 partners'. Compounds that disrupt such interactions may be useful in regulating the activity of the target protein, especially mutant target proteins. Such compounds may include, but are not limited to, molecules such as antibodies, peptides, and the like.

30       The basic principle of the assay systems used to identify compounds that interfere with the interaction between the target protein and its cellular or extracellular binding partner or partners involves preparing a reaction mixture 35 containing the target protein, and the binding partner

under conditions and for a time sufficient to allow the two to interact and bind, thus forming a complex. In order to test a compound for inhibitory activity, the reaction mixture is prepared in the presence and absence 5 of the test compound. The test compound may be initially included in the reaction mixture, or may be added at a time subsequent to the addition of target protein and its cellular or extracellular binding partner. Control reaction mixtures are incubated without the test compound 10 or with a placebo. The formation of any complexes between the target protein and the cellular or extracellular binding partner is the detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, 15 indicates that the compound interferes with the interaction of the target protein and the interactive binding partner. Additionally, complex formation within reaction mixtures contains the test compound and a mutant target protein. This comparison may be important in 20 those cases wherein it is desirable to identify compounds that disrupt interactions of mutant but not normal target proteins.

The assay for compounds that interfere with the 25 interaction of the target and binding partners can be conducted in a heterogeneous or homogenous format. Heterogeneous assays involve anchoring either the target protein or the binding partner onto a solid phase and detecting complexes anchored on the solid phase at the 30 end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the 35 compounds being tested. For example, test compounds that interfere with the interaction between the target protein

and the binding partners, e.g. by competition, can be identified by conducting the reaction in the presence of the test substance, i.e. by adding the test substance to the reaction mixture prior to or simultaneously with the target protein and interactive cellular or extracellular binding partner. Alternatively, test compounds that disrupt pre-formed complexes, e.g. compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a heterogeneous assay system, either the target protein or the interactive cellular or extracellular binding partner, is anchored onto a solid surface, while the non-anchored species is labelled, either directly or indirectly. In practice, microtiter plates are conveniently utilised. The anchored species may be immobilised by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the target gene product or binding partner and drying. Alternatively, an immobilised antibody specific for the species to be anchored may be used to anchor the species to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the partner of the immobilised species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (e.g. by washing) and any complexes formed will remain immobilised on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a

number of ways. Where the non-immobilised species is pre-labelled, the detection of label immobilised on the surface indicates that complexes were formed. Where the non-immobilised species is not pre-labelled, an indirect label can be used to detect complexes anchored on the surface, e.g. using a labelled antibody specific for the initially non-immobilised species (the antibody, in turn, may be directly labelled or indirectly labelled with a labelled anti-Ig antibody). Depending on the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt pre-formed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected, e.g. using an immobilised antibody specific for one of the binding components to anchor any complexes formed in solution, and a labelled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds which inhibit complex or which disrupt pre-formed complexes can be identified.

In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a pre-formed complex of the target protein and the interactive cellular or extracellular binding partner is prepared in which either the target protein or its binding partners is labelled, but the signal generated by the label is quenched due to complex formation (see, e.g. US Pat. No. 4,109,496 by Rubenstein which utilises this approach for immunoassays). The addition of a test substance that competes with and displaces one of the species from the

pre-formed complex will result in the generation of a signal above background. In this way, test substances, which disrupt target protein/cellular or extracellular binding partner interaction, can be identified.

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In a particular embodiment, the target protein can be prepared for immobilisation using recombinant DNA techniques. For example, the target protein gene coding region can be fused to a glutathione-S-transferase (GST) gene using a fusion vector, such as pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. The interactive cellular or extracellular binding partner can be purified and used to raise a monoclonal antibody, using methods routinely practised in the art and described above. This antibody can be labelled with the radioactive isotope  $^{125}\text{I}$ , for example, by methods routinely practised in the art. In a heterogeneous assay, e.g. the GST-target protein gene fusion protein can be anchored to glutathione-agarose beads. The interactive cellular or extracellular binding partner can then be added in the presence or absence of the test compound in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labelled monoclonal antibody can be added to the system and allowed to bind to the complexed components. The interaction between the target protein and the interactive cellular or extracellular binding partner can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by the test compound will result in a decrease in measured radioactivity.

35 Alternatively, the GST-target protein gene fusion protein

and the interactive cellular or extracellular binding partner can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test compound can be added either during or after the species are 5 allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again the extent of inhibition of the target protein/binding partner interaction can be detected by adding the labelled antibody and measuring 10 the radioactivity associated with the beads.

In another embodiment of the invention, these same techniques can be employed using peptide fragments that correspond to the binding domains of the target protein 15 and/or the interactive cellular or extracellular binding partner (in cases where the binding partner is a protein), in place of one or both of the full length proteins. Any number of methods routinely practised in the art can be used to identify and isolate the binding 20 sites. These methods include, but are not limited to, mutagenesis of the gene encoding one of the proteins and screening for disruption of binding in a co- immunoprecipitation assay. Compensating mutations in the 25 gene encoding the second species in the complex can then be selected. Sequence analysis of the genes encoding the respective proteins will reveal the mutations that correspond to the region of the protein involved in interactive binding. Alternatively, one protein can be anchored to a solid surface using methods described in 30 this section above, and allowed to interact with and bind to its labelled binding partner, which has been treated with a proteolytic enzyme, such as trypsin. After washing, a short, labelled peptide comprising the binding domain may remain associated with the solid material, 35 which can be isolated and identified by amino acid

sequencing. Also, once the gene coding for the cellular or extracellular binding partner is obtained, short gene segments can be engineered to express peptide fragments of the protein, which can then be tested for binding activity and purified or synthesised.

For example, and not by way of limitation, a target protein can be anchored to a solid material as described above, in this Section by making a GST-target protein gene fusion protein and allowing it to bind to glutathione agarose beads. The interactive cellular or extracellular binding partner can be labelled with a radioactive isotope, such as  $^{35}\text{S}$ , and cleaved with a proteolytic enzyme such as trypsin. Cleavage products

can then be added to the anchored GST-target protein gene fusion protein and allowed to bind. After washing away unbound peptides, labelled bound material, representing the cellular or extracellular binding partner binding domain, can be eluted, purified and analysed for amino acid sequence by well-known methods. Peptides so identified can be produced synthetically or fused to appropriate facilitative proteins using recombinant DNA technology.

In a further embodiment, cells that contain and express target gene sequences which encode target proteins and, further, exhibit cellular phenotypes associated with an insulin resistance disorder, may be utilised to identify compounds that exhibit an ability to ameliorate insulin resistance disorder symptoms. Cellular phenotypes, which may indicate an ability to ameliorate insulin resistance disorders, may include, for example, inhibition of glucose utilisation or insulin mediated glucose uptake by adipocytes.

Further, the fingerprint pattern of protein expression of cells of interest may be analysed and compared to the normal, insulin sensitive fingerprint pattern. Those compounds which cause cells exhibiting insulin resistance disorder-like cellular phenotypes to produce a fingerprint pattern more closely resembling a normal fingerprint pattern for the cell of interest may be considered candidates for further testing regarding an ability to ameliorate insulin resistance disorder symptoms.

Cells which can be utilised for such assays may, for example, include non-recombinant cell lines, such as preadipocyte cell lines such as 3T3-L1 and TA1 mouse preadipocyte cell lines, liver cell lines, such as the Hepal-6 mouse liver cell line, and the HepG2 human liver cell line.

Further, cell lines which may be used for such assays may also include recombinant, transgenic cell lines. For example, the insulin resistant disorder animal models of the invention discussed above, may be used to generate cell lines, containing one or more cell types involved in insulin resistance disorders, that can be used as cell culture models for this disorder. While primary cultures derived from the insulin resistant disorder transgenic animals of the invention may be utilised, the generation of continuous cell lines is preferred. For examples of techniques which may be used to derive a continuous cell line from the transgenic animals, see Small, et al., 1985, Mol. Cell Biol. 5: 642-648.

Alternatively, cells of a type known to be involved in insulin resistant disorders may be transfected with sequences capable of increasing or decreasing the amount

of target protein within the cell. For example, gene sequences of target proteins may be introduced into, and overexpressed in, the genome of the cell of interest, or, if endogenous gene sequences of the target protein are present, they may either be overexpressed or, alternatively, be disrupted in order to underexpress or inactivate target protein expression.

In order to overexpress a gene sequence of a target protein, the coding portion of the target gene sequence may be ligated to a regulatory sequence, which is capable of driving gene expression in the cell type of interest. Such regulatory regions will be well known to those of skill in the art, and may be utilised in the absence of undue experimentation.

For underexpression of an endogenous target protein the gene sequence may be isolated and engineered such that when reintroduced into the genome of the cell type of interest, the endogenous target gene alleles will be inactivated. Preferably, the engineered target gene sequence is introduced via gene targeting such that the endogenous target sequence is disrupted upon integration of the engineered target gene sequence into the cell's genome.

Transfection of target protein gene sequence nucleic acid may be accomplished by utilising standard techniques. See, for example, Ausebel, 1989, *supra*. Transfected cells should be evaluated for the presence of the recombinant target gene sequences, for expression and accumulation of target gene mRNA, and for the presence of recombinant target protein production. In instances wherein a decrease in target protein expression is desired, standard techniques may be used to demonstrate

whether a decrease in endogenous target gene expression and/or in target protein production is achieved.

5 In a further embodiment, animal-based model systems of insulin resistance disorders may be used for screening including, but are not limited to, non-recombinant and engineered transgenic animals.

10 Non-recombinant animal models for insulin resistance disorders may include, for example, genetic models. Such genetic insulin resistance models may include, for example, mouse models of non-insulin dependent diabetes and/or obesity such as mice homozygous for the autosomal recessive ob, db, or tub alleles.

15 Non-recombinant, non-genetic animal models of insulin resistance disorder may include, for example, rats or mice fed on a diet containing a large amount of fat. Such diets could be synthetic diets in which the fat content (by calorific value) is more than 50%.

20 Alternative human foods with a high fat content, such as salami and butter, may be provided to the animals.

25 Additionally, animal models exhibiting insulin resistance disorder-like symptoms may be engineered by utilising, for example, the gene sequences of target proteins such as those described above, in conjunction with techniques for producing transgenic animals that are well known to those of skill in the art. For example, gene sequences of target proteins may be introduced into, and overexpressed in, the genome of the animal of interest, or, if endogenous gene sequences of target proteins are present, they may either be overexpressed or, alternatively, may be disrupted in order to underexpress or inactivate gene expression of target proteins.

In order to overexpress the target gene sequence of a target protein, the coding portion of the target gene sequence may be ligated to a regulatory sequence, which is capable of driving gene expression in the animal and cell type of interest. Such regulatory regions will be well known to those of skill in the art, and may be utilised in the absence of undue experimentation.

For underexpression of an endogenous gene sequence of a target protein, such a sequence may be isolated and engineered such that when reintroduced into the genome of the animal of interest, the endogenous gene alleles of the target protein will be inactivated. Preferably, the engineered gene sequence of the target protein is introduced via gene targeting such that the endogenous sequence is disrupted upon integration of the engineered target gene sequence into the animal's genome.

Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, mini-pigs, goats and non-human primates, e.g. baboons, squirrels, monkeys and chimpanzees may be used to generate insulin resistant disorder animal models.

Any techniques known in the art may be used to introduce a target gene transgene of a target protein into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, US Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, Proc. Nat., Acad. Sci., USA 82: 6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, Cell 56: 313-321); electroporation of embryos (Lo, 1983, Mol. Cell Biol. 3: 1803-1814); and sperm-mediated gene transfer

(Lavitrano et al., 1989, Cell 57: 717-723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animalsm Intl. Rev. Cytol. 115: 171-229, which is incorporated by reference herein in its entirety.

5 The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals that carry the transgene in some, but not all their cells, i.e. mosaic animals (see, for example, 10 techniques described by Jakobovits, 1994, Curr. Biol. 4: 761-763). The transgene may be integrated as a single transgene or in concatamers, e.g. head-to-head tandems or head-to-tail tandems. The transgene may also be 15 selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko, M. et al., 1992, Proc. Natl. Acad. Sci. USA 89: 6232-6236). The regulatory sequences required to such a cell-type specific activation will 20 depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

When it is desired that the target gene transgene be 25 integrated into the chromosomal site of the endogenous target gene, gene targeting is preferred. Briefly, when such a technique is to be utilised, vectors containing some nucleotide sequences homologous to the gene of the endogenous target protein of interest are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the 30 function of, the nucleotide sequence of the endogenous target gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene of interest in only that cell type, by following, for example, the teaching of Gu et al. (Gu, 35 H. et al., 1994, Science 265: 103-106). The regulatory

sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

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Once transgenic animals have been generated, the expression of the recombinant target gene and protein may be assayed utilising standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyse animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, in situ hybridisation analysis, and RT-PCR. Samples of target protein-expressing tissue may also be evaluated immunocytochemically using antibodies specific for the transgene protein of interest.

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The target protein transgenic animals that express target gene mRNA or target protein transgene peptide (detected immunocytochemically, using antibodies directed against target protein epitopes) at easily detectable levels should then be further evaluated to identify those animals which display characteristic insulin resistant disorder-like symptoms. Such symptoms may include, for example, obesity, glucose intolerance and/or non-insulin dependent diabetes. Additionally, specific cell types within the transgenic animals may be analysed and assayed for cellular phenotypes characteristic of insulin resistant disorders. Such cellular phenotypes may include, for example, abnormal adipocyte differentiation (e.g. abnormal preadipocyte/adipocyte differentiation) and metabolism. Further, such cellular phenotypes may

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include an assessment of a particular cell types fingerprint pattern of expression and its comparison to known fingerprint expression profiles of the particular cell type in animals exhibiting insulin resistance disorders. Such transgenic animals serve as suitable model systems for insulin resistance disorders.

Once target protein transgenic founder animals are produced (i.e. those animals which express target proteins in cells or tissues of interest and which, preferably, exhibit symptoms of insulin resistance disorders), they may be bred, inbred, outbred or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include, but are not limited to, outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound target protein transgenics that transgenically express the target protein of interest at higher levels because of the effects of additive expression of each target gene transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the possible need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; breeding animals to different inbred genetic backgrounds so as to examine effects of modifying alleles on expression of the target protein and the development of insulin resistance disorder-like symptoms. One such approach is to cross the target protein transgenic founder animals with a wild type strain to produce an F1 generation that exhibits insulin resistance disorder-like symptoms, such as glucose intolerance, hyperinsulinaemia, non-insulin dependent diabetes and

obesity. The F1 generation may then be inbred in order to develop a homozygous line, if it is found that homozygous target protein transgenic animals are viable.

5        Diagnostic assays for DEPs and PPs

It will be obvious to those skilled in the art that the causes and nature of the development of insulin resistance will not be the same in all individuals. Thus the pattern of DEPs will not be identical in all individuals. These different aetiologies of insulin resistance and patterns of DEPs will lead to the discovery of different optimal therapies for each of the different causes of insulin resistance. Thus, diagnostic assays that will identify the DEP pattern in an insulin resistance subject including non-insulin dependent diabetes will permit a diagnosis of the optimal therapy. Furthermore, such a diagnostic assay will also allow a prognosis of the value of any treatment.

20        The sample used in such diagnostic assay could be of any appropriate cellular material involved in insulin resistance. Besides those listed above in connection with establishing a paradigm, they can be monocytes or lymphocytes. Ideally, the sample will be a readily available body fluid such as blood or urine in which fingerprint proteins are secreted and their concentration reflects the mature and degree of the insulin resistance.

25        One preferred method of assaying (quantifying, estimating or detecting) the presence of a DEP or PP in a sample from an appropriate body tissue comprises reacting the sample with a specific binding partner, preferably an antibody, specific for the DEP, to form a reaction product, typically a complex between the DEP and its antibody, and determining the presence or amount of the

reaction product. Preferably they are affinity assays or immunoassays.

Examples of suitable such antibodies are those described above. Any known method of immunoassay may be used. An antibody capture assay is preferred. Here, the test sample is allowed to bind to a solid phase, such as a well of a plastics microtitre plate, and the anti-DEP or PP antibody, is then added and allowed to bind. After washing away unbound material, the presence or amount of antibody bound to the solid phase is determined using a labelled second antibody, as well known in the art. The second antibody may be provided within the same molecule as the first or as a separate molecule, usually an anti-Ig of the first antibody.

Alternatively, a competition assay could be performed between the sample and a labelled peptide having the same sequence as the relevant epitope of the DEP or PP, these two antigens being in competition for a limited amount of antibody bound to a solid support. The labelled peptide could be pre-incubated with the antibody on the solid phase, whereby the DEP or PP in the sample displaces part of the peptide bound to the antibody.

Alternatively, the two antigens are allowed to compete in a single co-incubation with the antibody. After removal of unbound antigen from the support by washing, the amount of label attached to the support is determined and the DEP or PP in the sample is detected or measured by a reference to standard titration curves established previously.

The substrate for the enzyme may be colour-forming, fluorescent or chemiluminescent.

It is highly preferable to use an amplified form of assay, whereby an enhanced "signal" is produced from a relatively low level of protein to be detected. One particular well known form of amplified immunoassay is 5 enhanced chemiluminescent (ECL) assay. Here, the antibody is preferably labelled with horseradish peroxidase, which participates in a chemiluminescent reaction with luminol, a peroxide substrate and a compound which enhances the intensity and duration of the 10 emitted light, typically 4-iodophenol or 4-hydroxycinnamic acid.

Another preferred form of amplified immunoassay is immuno-PCR. In this technique, the antibody is 15 covalently linked to a molecule of arbitrary DNA comprising PCR primers, whereby the DNA with the antibody attached to it is amplified by the polymerase chain reaction. See E. R. Hendrickson *et al.*, Nucleic Acids Research, 1995: 23, 522-529 or T. Sano *et al.*, 1995, in 20 "Molecular Biology and Biotechnology" ed. Robert A. Meyers, VCH Publishers, Inc., pages 458 - 460. The signal is read out as before.

The assay reagents may be formulated as a kit, usually 25 together with an appropriate standard which is the same as the protein to be assayed. Thus, a kit of the invention preferably comprises a directly or indirectly labelled antibody to the DEP or PP to be assayed, together with the DEP or PP itself, as standard. 30 Optionally it may include a buffer, substrate (where the label is an enzyme) and (where the antibody is attached to DNA) PCR primers for the DNA.

Inhibition of expression, synthesis or activity of target  
proteins

Where the target protein is over-expressed in the insulin-resistant state, it will be desirable to inhibit its expression, production or activity *in vivo*. There are many ways of doing this. Examples are:

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A. Administering a compound which will bind to the target protein *in vivo*, for example a peptide which corresponds to the soluble extracellular portion of a transmembrane receptor for the target protein or a compound (peptide or non-peptide) which mimics the action of the transmembrane receptor. Preferably the compound is a non-peptide organic compound of low molecular weight, e.g. below 600. However, it may also be a peptide of sufficient length to bind to the binding site of the target protein so as to block its interaction with the transmembrane receptor.

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B. Administering an antibody specific to the target protein, preferably a monoclonal antibody raised against it.

C. The target proteins may, *in vivo*, interact with one or more cellular or extracellular macromolecular binding partners, e.g. proteins or nucleic acids. Compounds that disrupt such interactions may be useful in regulating the activity of the target protein. Again, these are preferably low molecular weight organic compounds or peptides as described above under A.

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D. Synthesis of the target protein can be inhibited at the gene level. The corresponding gene can be identified as described above under "Identification and characterisation of DEPs". Antisense, ribozyme or triple helix-forming molecules can be designed to reduce or inhibit expression of the target protein gene.

Techniques for the production and use of such molecules are well known in the art.

Further details of ways of identifying the relevant 5 agents in A-D are given in an Appendix 1 at the end of the description.

The synthesis of the target protein may also be inhibited by agents that act on regulatory regions of the target 10 protein gene sequence or by agents that block the production or action of the natural factors that up-regulate the target protein in the insulin resistant state.

15 **Methods for increasing the level or activity of a target protein**

Where the target protein is under-expressed in the insulin-resistant state it will be desirable to enhance its expression, production or activity in vivo or to 20 provide replacement protein. There are many ways of doing this. Examples are:

A. Administering recombinant target protein at a sufficient level to alleviate the insulin resistance 25 disorder symptoms to a patient exhibiting such symptoms.

B. Administering a compound which will bind to the regulatory sequence of the target protein gene and affect an increase in the synthesis of the target protein. Such 30 compounds are preferably non-peptide organic compound of low molecular weight below 600.

C. Administering a compound which will increase the stability of the target protein mRNA or will inhibit the 35 rate of turnover of the target protein.

D. Patients may be treated by gene replacement therapy. One or more copies of a normal target protein gene or a portion of the gene that directs the production of a normal target protein with target protein gene function, may be inserted into cells, using vectors which include adenovirus, adeno-associated virus, and retrovirus vectors, in addition to other particles that introduce DNA into cells, such as liposomes. Additionally, techniques such as those described above may be used for the introduction of normal target protein gene sequences into human cells.

Cells, preferably autologous cells, containing normal target protein gene sequences may then be introduced or reintroduced into the patient at positions which allow for the amelioration of insulin resistance disorder symptoms. Such cell replacement techniques may be preferred, for example, when the target protein is a secreted, extracellular protein.

Additionally, antibodies may be administered which specifically bind to a target protein and by binding, serve to, either directly or indirectly, activate the target protein function. Such antibodies may be any of those described above in relation to DEPs. They may be administered by the techniques described above.

#### Methods of administration

The agents (a compound or compounds) selected by the screening methods described above can be administered to a patient at therapeutically effective doses to treat or alleviate insulin resistance disorders. A therapeutically effective dose refers to that amount of the agent sufficient to result in alleviation of symptoms of insulin resistance disorder, including non-insulin

dependent diabetes.

**Effective dose**

5 The toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g. for determining by ED<sub>50</sub> (the dose that effects a 50% improvement in insulin sensitivity) and by determining the ED<sub>50</sub> of any side-effects (toxicity - TD<sub>50</sub>). The dose 10 ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio TD<sub>50</sub>/ED<sub>50</sub>. Compounds, which exhibit large therapeutic indices, are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to 15 design a delivery system that targets such compounds to the site of affected tissue in order to minimise potential damage to cells and, thereby, reduce side effects.

20 The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The 25 dosage may vary within this range depending upon the dosage form employed and the route of administration used.

30 For the treatment of humans, a typical dose of the agent per day would be 0.01mg to 4g, preferably 0.01 - 400mg and more preferably 0.1 to 10mg, all based on 70kg bodyweight, per day, given either at a single time or at up to 8 times per day preferably no more than 4 times per day.

Formulations

The agent selected by the screening method may be a single compound or a mixture or combination of two compounds. The terms "compound" will be used in this 5 section for brevity, it being understood to include singular and plural. The compound selected by the screening method of the present invention may be formulated in conventional manner, normally as a pharmaceutical composition comprising the compound and 10 one or more physiologically acceptable carriers, diluents, adjuvants or excipients. Thus, the compound and its physiologically acceptable salts and solvates may be formulated for administration by inhalation or 15 insufflation (either through the mouth or the nose) or oral, buccal, parenteral and rectal administration.

For oral administration, the pharmaceutical compositions 20 may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g. pre-gelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methyl cellulose); fillers (e.g. lactose, 25 microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g. magnesium stearate, talc or silica); disintegrants (e.g. potato starch or sodium starch glycollate); or wetting agents (e.g. sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral 30 administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid 35 preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g. sorbitol syrup, cellulose derivatives or

hydrogenated edible fats); emulsifying agents (e.g. lecithin or acacia); and preservatives (e.g. methyl or propyl p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavouring, 5 colouring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

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For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

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For administration by inhalation, the compound for is conveniently delivered in the form of an aerosol spray presentation from pressurised packs or a nebuliser, with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane,

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dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurised aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g. gelatin, for use in an inhaler or insufflator may be 25 formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

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The compound may be formulated for parenteral administration by injection, e.g. by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g. in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and 35 may contain formulatory agents such as suspending,

stabilising and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g. sterile pyrogen-free water, before use.

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The compound may also be formulated in rectal compositions such as suppositories or retention enemas, e.g. containing conventional suppository bases such as cocoa butter or other glycerides.

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In addition to the formulations described previously, the compound may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation, for example, subcutaneously or 15 intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, 20 as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device, which may contain one or more unit dosage forms containing the active ingredient. The pack 25 may, for example, comprise metal or plastic foil, such as blister pack. The pack or dispenser device may be accompanied by instructions for administration.

#### Diagnosis of insulin resistance disorders

30 The invention includes the diagnosis of the nature or extent of an insulin resistance disorder, pre-disposition to an insulin disorder, for monitoring the efficacy of the selected screened agents during, for example, clinical trials thereof, and for monitoring patients 35 undergoing clinical evaluation for the treatment of such

insulin resistance disorders.

The method can be applied to subjects who have received treatment or not. They may be in a diabetic or pre-diabetic condition.

5 The method comprises establishing an appropriate paradigm, as described above, obtaining a sample of tissue from the patient (human or animal), especially from monocytes or lymphocytes, or a suitable body fluid and assaying the sample for the presence or amount of expression in the tissue of one or more DEPs for which the paradigm has been established. Such DEPs may be described as fingerprint proteins. The observed pattern 10 and/or amount of expression is then related to clinical information which has been obtained previously, so as to 15 establish "standards".

20 The methods of obtaining clinical information include conventional methods such as measurements of the effectiveness of insulin in mediating its normal response on a cellular process such as glucose or amino acid uptake into a primary cell preparation such as 25 lymphocytes taken from the patient or to an *in vivo* response. In addition techniques such as measurements of glucose/insulin ratios in humans or in whole animals or glucose tolerance tests or the euglycaemic hyperinsulinaemic clamp procedure may be used.

30 NIDDM may be detected and the efficacy of treatment monitored by methods and parameters identified by such bodies as the World Health Organisation, the International Diabetes Federation and the American Diabetes Association.

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Methods and reagents used to assay DEPs and PPs, as described above, may be used in laboratory and clinical trials. Specifically, such methods and reagents may be used, for example, for: (1) the detection of the presence of target protein mutations, or (2) the detection of either an over- or an under-abundance of target protein relative to the non-insulin resistance disorder state.

5 The following Examples illustrate the invention.

10 Example 1

Mouse treatment protocol

Lean and obese female C57 Bl/6J ob/ob mice were given BRL 49653, rosiglitazone, 10mg/kg/day, by oral gavage for 7 days. This treatment produced significant improvement in oral glucose tolerance and insulin sensitivity in ob/ob mice, but had no effect in the lean litter mates. Liver tissues were taken from the above 8 weeks-old lean and ob/ob mice. Non-fasting animals were anaesthetized with 50% "Hyponovel" and 50% "Hyponorm" and then killed with 0.9% saline buffer. The liver was perfused with snap-frozen between tongs in liquid nitrogen, dissected, for 48 hours and crushed in a mortar in presence of liquid nitrogen. The resulting dried powder was stored at -80°C until analysis.

Protein solubilisation

For analytical 2-D-PAGE, 200 micrograms of dried liver was mixed with 60 microlitres of a solution containing urea (8 M), CHAPS (4% w/v), Tris (40 mM), DTE (65 mM), SDS (0.05% w/v) and a trace of Bromophenol Blue. A weighed portion of the final diluted sample (100 micrograms of protein) was loaded into a sample cup at

the cathodic end of the IPG gels.

**First dimension electrophoresis**

5 A non-linear immobilized pH gradient of IPG strips (3.5-  
10.0 NL IPG 18 cm) was used as the first dimension. It  
offered high resolution, great reproducibility and  
allowed high protein loads. Based on specifications of  
10 the Geneva University Hospital, the non-linear pH  
gradient strips were prepared by Amersham-Pharmacia  
Biotechnology AB and are commercially available. The  
strips were 3 mm wide and 180 mm long.

15 Hydration of the IPG strips was performed overnight in a  
Pharmacia reswelling cassette with 25 ml of a solution of  
urea (8 M), CHAPS (2% w/v), DTE (10 mM), Resolyte pH 3.5-  
10 (2% v/v) and a trace of Bromophenol Blue.

20 When the rehydration cassette had been thoroughly emptied  
and opened, the strips were transferred to the Pharmacia  
strip tray. After placing IPG strips, humid electrode  
wicks, electrodes and sample cups in position, the strips  
and cups were covered with low viscosity paraffin oil.  
25 Samples were applied in the cups at the cathodic end of  
the IPG strips in a slow and continuous manner, without  
touching the gel.

30 The voltage was linearly increased from 300 to 3500 V  
during 3 hours, followed by 3 additional hours at 3500 V,  
whereupon the voltage was increased to 5000 V. A total  
volt.hour product of 100 kvh was used in an overnight  
run.

**Second dimension of the electrophoresis**

35 After the first dimension run, the IPG strips were  
equilibrated in order to resolubilise the proteins and to

reduce -S-S- bonds. The strips were thus equilibrated within the strip tray with 100 ml of a solution containing Tris-HCl (50 mM) pH 6.8, urea (6 M), glycerol (30% v/v), SDS (2% w/v) and DTE (2% w/v) for 12 min. The SH groups were subsequently blocked with 100 ml of a solution containing Tris-HCl (50 mM) pH 6.8, urea (6 M), glycerol (30% v/v), SDS (2% w/v), iodoacetamide (2.5% w/v) and a trace of Bromophenol Blue for 5 min.

10 In the second dimension run, a vertical gradient slab gel with the Laemmli-SDS-discontinuous system was used with some small modifications, which may be summarised as follows:

15 • Gels are not polymerised in the presence of SDS. This seems to prevent the formation of micelles which contain acrylamide monomer, thus increasing the homogeneity of pore size and reducing the concentration of unpolymerised monomer in the 20 polyacrylamide. The SDS used in the gel running buffer is sufficient to maintain the necessary negative charge on proteins.

25 • Piperazine-diacrylyl (PDA) is used as crosslinker. This is believed to reduce N-terminal protein blockage, gives better protein resolution, and reduces diammine silver staining background.

30 • Sodium thiosulphate is used as an additive to reduce background in the silver staining of gels.

• The combination of the IPG strip and agarose avoids the need for a stacking gel.

The gel composition and dimensions were as follows:

Dimensions: 160 x 200 x 1.5 mm

Resolving gel: Acrylamide/PDA (9-16% T / 2.6% C)

35 Stacking gel: No stacking

Leading buffer: Tris-HCl (0.375 M) pH 8.8  
Trailing buffer: Tris-glycine-SDS (25 mM-198 mM-  
0.1% w/v) pH 8.3  
Additives: Sodium thiosulphate (5 mM)  
5 Polymerisation agents: TEMED (0.05%) APS (0.1%).

The gels were poured until 0.7 cm. from the top of the plates and over-layered with sec-butanol for about two hours. After the removal of the overlay and its

10 replacement with water the gels were left overnight.

After the equilibration, the IPG gel strips were cut to size. Six mm were removed from the anodic end and 14 mm from the cathodic end. The second dimension gels were

15 over-layered with a solution containing agarose (0.5% w/v) and Tris-glycine-SDS (25 mM-198 mM-0.1% w/v) pH 8.3 heated at about 70° C and the IPG gel strips were immediately loaded through it.

20 The gel was run at 8-12°C for 5 hours at a constant current of 40 mA/gel. The voltage is non-limiting, but usually requires 100 to 400 V.

#### Staining

25 Silver staining, which is 100-fold more sensitive than Coomassie Brilliant Blue staining, was used (except where otherwise stated). Thus, the 2-DGE gels were stained with an ammoniacal silver staining as follows:

30 All steps were performed on an orbital shaker at 36 rpm.  
Step 1: At the end of the second dimension run, the gels were removed from the glass plates and washed in deionized water for 5 min.

Step 2: The gels were soaked in ethanol: acetic acid: water (40: 10: 50 volume ratio) for 1 hour.

35

Step 3: The gels were soaked in ethanol: acetic acid: water (5: 5: 90 volume ratio) for 2 hours or overnight.

Step 4: They were washed in deionized water for 5 min.

5 Step 5: They were soaked in a solution containing glutaraldehyde (1% v/v) and sodium acetate (0.5 M) for 30 min.

Step 6: They were washed 3 times in deionized water for 10 min.

10 Step 7: In order to obtain homogeneous dark brown staining of proteins, gels were soaked twice in a 2,7-naphthalenedisulphonic acid solution (0.05% w/v) for 30 min.

Step 8: The gels were then rinsed 4 times in deionized water for 15 min.

15 Step 9: The gels were stained in a freshly made ammoniacal silver nitrate solution for 30 minutes. To prepare 750 ml of this solution, 6 g of silver nitrate were dissolved in 30 ml of deionized water, which was slowly mixed into a solution containing 160 ml of water, 20 10 ml of concentrated ammonia (25%) and 1.5 ml of sodium hydroxide (10N). A transient brown precipitate might form. After it cleared, water was added to give the final volume.

Step 10: After staining, the gels were washed 4 times in deionized water for 4 min.

25 Step 11: The images were developed in a solution of citric acid (0.01% w/v) and formaldehyde (0.1% v/v) for 5 to 10 min.

30 Step 12: When a slight background stain appeared, development was stopped with a solution of Tris (5% w/v) and acetic acid (2% v/v).

#### Scanning of the gels

35 The Laser Densitometer (4000 x 5000 pixels; 12 bits/pixel) from Molecular Dynamics and the GS-700 from

Bio-Rad have been used as scanning devices. These scanners were linked to "Sparc" workstations and "Macintosh" computers.

5       Quantitative image analysis of the gels using "Melanie II"

Two-dimensional polyacrylamide gels may be digitised and analysed by computer to allow quantitative image analysis and automatic gel comparison. Since the 2-D-PAGE 10 technique was first developed in 1975 several computer systems have been manufactured, mainly by academic 2-D-PAGE related laboratories. In the present work, "Melanie II", developed at the University Hospital of Geneva was used. It is available for "Unix" workstations, as well as 15 for "Power Macintosh" and IBM-compatible computers.

Ob/obb and lean mice spot detection, quantitation and matching, gel image extraction, zooming, warping and printing as well as gel stacking and flipping were 20 carried out with the "MelView" program.

The images were then classified in four classes; lean control, ob/ob control, lean treated and ob/ob treated. Differential analysis and the Student T test, using the 25 relative abundance of each spot (% volume), allowed the detection of significant ( $p<0.01$ ) over- and under-expressed polypeptides.

Preparative 2-D-PAGE

30       The analytical 2-DGE described above was repeated, with the following changes. Four mg of dried liver was mixed with 450 microlitres of the solubilisation solution and loaded into the IPG strips by in-gel rehydration. After the first dimension run the strips were equilibrated 35 using 3 ml of each buffer per groove.

Protein electroblotting

The blotting of proteins separated by 2-D-PAGE onto polyvinylidene difluoride (PVDF) membranes has enabled the identification and characterisation of proteins from complex biological samples. Transfer of the proteins can be carried out using several methods such as vacuum, capillary or electric field. Electroblotting, using vertical buffer tanks or a semi-dry method, is preferred. Both techniques can use the 3-[cyclohexamino]-1-propanesulfonic acid (CAPS) transfer buffer. Gloves must be worn and all filter papers should be washed three times for 3 min in water and three times in transfer buffer. These two steps are important in order to avoid any protein or amino acid contamination.

The procedure was as follows. After second-dimensional electrophoresis, the gels were soaked in deionized water for 3 min. Then they were equilibrated in a solution containing 10 mM CAPS pH 11 for 30 min. At the same time, PVDF membranes were wetted in methanol for 1 min and equilibrated in a solution containing 10 mM CAPS pH 11 and methanol (10% v/v) also for 30 min.

Electroblotting was carried out in a semi-dry apparatus with a solution containing 10 mM CAPS pH 11 and methanol (20% v/v anodic side; 5% v/v cathodic side) at 1 mA/cm<sup>2</sup> constant current for 3 hours at 15°C.

Protein detection on PVDF membranes

Amido Black and Coomassie Brilliant Blue R-250 were used instead of silver staining to visualise proteins on PVDF membranes and are compatible with the ensuing post-separation analysis. Thus, in another 2-DGE run, after electrotransfer, the PVDF membranes were stained in a solution containing Amido Black (0.5% w/v), isopropanol

(25% v/v) and acetic acid (10% v/v) for 2 min. Destaining was done by several soakings in deionized water.

5 In another run, after electrotransfer, the PVDF membranes were stained in a solution containing Coomassie Brilliant Blue R-250 (0.1% w/v) and methanol (50% v/v) for 15 min. Destaining was done in a solution containing methanol (40% v/v) and acetic acid (10% v/v). The same method was used for preparative gels that did not need  
10 electrotransfer for further post-separation analysis, such as peptide mass fingerprinting.

15 The PVDF stained membranes were either air-dried or dried on a 3 mm thick plate onto an heating plate at 37 °C for 10 min.

#### Scanning

This was done as described above.

20 Protein identification

In amino sequence analysis by Edman degradation, amino acid derivatives are sequentially cleaved one at a time from the protein. Proteins with a chemically inaccessible alpha-amino group cannot be sequenced directly by this  
25 procedure and are termed N-terminally blocked. The best way to overcome the blocked proteins is to generate individual fragments by chemical or proteolytic cleavage.

30 Routinely, ten to twelve Edman degradation cycles were performed for each spot. A search in the SWISS-PROT database was made to detect identity to known protein sequences.

35 The Amido Black stained proteins were excised with a razor blade and N-terminal sequencing was performed using

an ABI model 473A or 477A microsequencer from Applied Biosystems equipped with "Problott" cartridges.

For internal sequencing, the spots of interest were 5 excised and soaked for two hours in a solution containing acetic acid (100 mM), methanol (10% v/v) and PVP-40 (1% v/v) at 37°C. After three washes in deionized water, the PVDF spots were cut into small pieces (about 1 mm<sup>2</sup>) and 10 incubated in 25 microlitres of a solution containing sodium phosphate (100 mM) pH 8.0 and lysyl endopeptidase (1 microgram). Following overnight digestion at room temperature, guanidine-HCl (28 mg) and DTT (100 micrograms) were added. After reduction for 2 hours at 37°C, the mixture was incubated for 30 min, at room 15 temperature, with 300 micrograms of iodoacetamide. The digestion solution was removed and kept. PVDF pieces were then extracted overnight with 25 microlitres of a solution containing isopropanol (70% v/v) and 20 trifluoroacetic acid (5% w/v). This elution solution was removed and the PVDF was washed twice with 60 microlitres of TFA (0.1% w/v). The digestion and elution solutions were pooled together with two final washes and this 25 mixture was separated by two-dimensional reverse phase HPLC and sequence determination performed.

#### Immunoblotting

PVDF membranes were first stained to visualise proteins, following which the immunodetection was undertaken. This 30 allowed matching of proteins detected with ECL against those detected with the non-specific protein stain through computer comparison of both images. The mechanical strength of PVDF was also exploited as the same 2-D gel can be used many times for different antibodies.

The whole procedure was carried out in a rotating oven at room temperature. The use of a nucleic acid glass hybridiser tube minimised the volumes and costs.

5     • The membranes were blocked in 10 ml of a solution of PBS (pH 7.2) and non-fat dry milk (5% w/v) for 30 min.

10    • The membranes were then incubated in 10 ml of a solution containing PBS-“Tween” 20 (0.5% v/v), non-fat dry milk (5% w/v) and the primary antibody/antibodies (1:100 or greater, depending on the antibody) for 2 hours.

15    • Three quick rinses were performed with 10 ml of PBS-“Tween” 20 (0.5% v/v) and then the membranes were washed for 3 x 10 min with 10 ml of PBS-“Tween” 20 (0.5% v/v).

20    • The membranes were incubated in 10 ml of a solution containing PBS-“Tween” 20 (0.5% v/v), non-fat dry milk (5% w/v) and the secondary peroxidase-conjugated antibody (1:1000; for example, if the primary antibody was sheep anti-mouse, then goat anti-sheep IgG was used as the secondary antibody) for 1 hour.

25    • Three quick rinses were performed with 10 ml of PBS-“Tween” 20 (0.5% v/v) and then the membranes were washed for 5 x 10 min with 10 ml of PBS-“Tween” 20 (0.5% v/v).

30    • After the last wash, the membranes were transferred to a clean glass plate and covered with 10 ml of developing solution (for example ECL from Amersham International or Roche Diagnostics) for 2 min.

35    • The excess developing solution was drained, the membranes were wrapped in “Saran” film and fixed in an X-ray film cassette with the proteins facing up.

• An X-ray film was then exposed in a dark room for

few seconds or up to several minutes.

**Peptide mass fingerprinting**

5 The 2-DGE method was repeated, but using a Coomassie blue stain. The 2-DGE spots were destained with 100 microlitres of 30% acetonitrile in 50 mM ammonium bicarbonate at 37°C for 45 min. The supernatant was discarded and the gel spots dried in a "SpeedVac" for 30 min. The gel spots were rehydrated with 25 microlitres of 10 a solution containing 0.2 micrograms of porcine trypsin and 50 mM ammonium bicarbonate for 2 hours at 35°C. Then the gel spots and supernatant were dried in a "SpeedVac" for 30 min, rehydrated with 20 microlitres of H<sub>2</sub>O for 30 min at 35°C and dried again for 30 min. Twenty 15 microlitres of a solution of 50 % of acetonitrile and 0.1 % of TFA was added to the spots and sonicated for 10 min.

20 Two microlitres of the supernatants was loaded in each well of a 96 or 400 MALDI target plate. The samples were air-dried. Then 2 microlitres of a solution containing 4 mg/ml of alpha-cyano-4-hydroxycinnamic acid, 50 % acetonitrile and 0.1 % TFA was added to each well and air-dried.

25 The peptide mixtures were analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Perseptive Biosystems Voyager Elite MALDI-TOF-MS) with a nitrogen laser (337 nm) and operated in reflectron delayed extraction mode.

30 Protein identification has been carried out using "PeptIdent" (<http://www.expasy.ch/sprot/peptident.html>). It is a tool that allows the identification of proteins using pI, relative molecular mass and peptide mass 35 fingerprinting data. Experimentally measured, user-

specified peptide masses were compared with the theoretical peptides calculated for all proteins in the SWISS-PROT/TREMBL databases.

5 **MS/MS sequencing**

When protein identification was not successful with the peptide mass fingerprinting procedure, the supernatant of digested spots was desalted in "ZipTip" C18 pipette tips (Millipore) and eluted with 50 % acetonitrile and 0.1 % TFA. Peptides were applied by nanoflow (in-house nanospray) sample introduction to a tandem mass spectrometer that consists of two quadrupoles and an orthogonal time of flight tube (Q-TOF) from MicroMass (UK). Fragment ion spectra were interpreted with the MOWSE database search  
[15 \(http://www.seqnet.dl.ac.uk/mowse.html\)](http://www.seqnet.dl.ac.uk/mowse.html).

**Data management: The mouse SWISS-2-DPAGE database**

SWISS-2-DPAGE is an annotated 2-D-PAGE database in which all the data are easily retrieved by computer programs and stored in a format similar to that of the SWISS-PROT Protein Sequence Database, one of the most updated and annotated protein sequence databases presently available. The SWISS-2-DPAGE database assembles data on proteins identified on various 2-D-PAGE maps. Each SWISS-2-DPAGE entry contains data on one protein, including mapping procedures, physiological and pathological data and bibliographical references, as well as several 2-D-PAGE images showing the protein location. Cross-references are provided to SWISS-PROT and, through the latter, to other databases (EMBL, Genbank, PROSITE, OMIM, etc). The database has been set up on the ExPASy World Wide Web server (<http://www.expasy.ch/>). Worldwide, scientists using similar 2-D-PAGE protocols (immobilized pH gradient as first dimensional separation) are now able to compare  
35

their images with SWISS-2-DPAGE maps.

**Results**

The following DEPs were found:

5

Group 1. LOM16, LOM17, LOM18, LOM20, LOM27, LOM28, LOM29, in which differential expression was observed by comparing lean controls with ob/ob controls. No differential expression of these "OM" proteins was seen when ob/ob untreated were compared with ob/ob treated. In other words, these proteins were not sensitive to rosiglitazone.

10

Group 2. LOMT19, LOMT21 - LOMT26, in which differential expression was observed by comparing lean controls with ob/ob controls and which were also differentially expressed when ob/ob controls were compared with ob/ob treated. After the treatment, the expression of each of these DEPs became similar to its expression in the lean control. In other words, the treatment appeared to restore most or all of the expression of that protein occurring in the normal animal. In each case, lean treated were compared with lean controls and no change in the expression of the DEP was observed. In other words, the differential expression appeared to be related to the treatment of the insulin resistant state in ob/ob mice and not merely to some side effect of rosiglitazone.

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Group 3. Only one DEP, LSEM30, falls into this group. It differs from Group 2 only in that when the lean treated were compared with lean controls, a change in the expression of this DEP was observed. Thus, this DEP might represent a potential side-effect of the rosiglitazone treatment. This information will be useful when other drugs are screened, to determine whether they

are similar or dissimilar to rosiglitazone in their action.

5 The drawings are presented to show the location of the DEPs by reference to Figure 1 and the differential expression in Figures 2 - 16, with Figure 17 showing a control protein for which no differential expression was observed.

10 The locations are shown in the maps of Figures 1A - 1E, in which Figure 1E shows the entire map from the 2-D-PAGE of liver tissue of lean mice. (It so happens that all the differentially expressed proteins identified are over-expressed in the lean control mice, relative to the ob/ob control mice.) The map is divided into four quadrants in 15 the manner:

Fig. 1A	Fig. 1B
Fig. 1C	Fig. 1D

20

in which the pI runs from left (low pI) to right (high pI) and the relative molecular mass from top to bottom, so that the quadrant of Figure 1A shows the spots 25 corresponding to proteins of the lowest pI and highest relative molecular mass and the quadrant of Figure 1D those of the highest pI and lowest relative molecular mass. The DEPs are marked as LOM16, LOM17, LOM18, LOMT19, LOM20, LOMT 21, LOMT22, LOMT23, LOMT24, LOMT25, 30 LOMT26, LOM27, LOM28, LOM29, LSEM30. The other spots identified are for reference purposes and have numbers of five digits preceded by "P" or "Q", corresponding to the SWISS-PROT accession number, thus linking the spots to their genes. The following Tables 1 and 2 list the

characteristics of the DEP spots in terms of relative volumes, areas, and optical densities and apparent pI and relative molecular mass.

5      **Table 1: DEP spots from liver of obese mice treated with rosiglitazone**

	ID	% Vol.	Area	% OD	pI	RMM
10	LOM16	0.177	1.470	1.460	6.35	160851
	LOM17	0.034	0.888	0.595	5.61	90000
	LOM18	0.059	0.858	0.959	6.54	82896
	LOM19	0.370	3.154	1.414	5.31	58000
15	LOM20	0.295	2.603	1.438	5.83	56422
	LOMT21	0.409	3.307	1.475	6.31	49972
	LOMT22	0.283	2.328	1.492	6.25	42937
	LOMT23	0.245	1.960	1.534	6.80	36508
20	LOMT24	0.293	2.419	1.462	6.83	32671
	LOMT25	0.388	3.277	1.439	6.26	26266
	LOMT26	0.298	2.664	1.398	5.10	24202
	LOM27	0.168	2.174	1.012	7.17	15283
	LOM28	1.495	10.596	1.655	9.07	13016
	LOM29	0.255	2.756	1.200	6.33	11946
	LSEM30	0.035	1.286	0.338	4.98	11731

Table 2: Reference spots from liver of obese control mice

	ID	% Vol.	Area	% OD	pI	RMM
5	P99015	0.122	1.102	1.286	6.31	160851
	P99015	0.177	1.470	1.460	6.35	160851
	P99015	0.161	0.766	1.076	6.17	160851
	P99015	0.098	1.041	1.252	6.22	160851
	P99015	0.157	1.715	1.302	6.26	160000
10	Q05920	0.085	1.133	1.050	6.16	121447
	Q05920	0.039	0.643	0.803	6.12	121447
	Q05920	0.201	2.266	1.281	6.21	120000
	P08113	0.023	0.306	0.981	4.87	90604
	P08113	0.082	0.827	1.214	4.88	89679
15	P08113	0.025	0.337	0.911	4.89	90000
	P99017	0.011	0.766	0.176	5.42	90604
	P99017	0.020	1.194	0.228	5.52	90000
	P99017	0.034	0.088	0.595	5.61	90000
	P99017	0.094	1.317	0.077	5.68	89359
20	P99017	0.020	0.888	0.298	5.77	90000
	P11499	0.033	0.766	0.669	5.02	82305
	P37040	0.020	0.674	0.465	5.28	71490
	P37040	0.008	0.429	0.226	5.25	71299
	P20029	0.030	0.735	0.553	4.99	70356
25	P20029	0.074	0.919	1.072	5.01	70169
	P20029	0.433	3.951	1.391	5.04	69426
	P38647	0.044	1.102	0.539	5.29	69426
	P38647	0.011	0.643	0.200	5.27	69426
	P38647	0.067	1.133	0.861	5.32	69057
30	P38647	0.266	2.909	1.273	5.34	68690
	P16627	0.027	1.164	0.317	5.25	67781
	P16627	0.066	0.949	0.970	5.27	67421
	P16627	0.367	3.675	1.304	5.30	67063
	P07724	0.022	0.888	0.343	5.68	66707
35	P07724	0.133	1.378	1.256	5.79	66529

	ID	% Vol.	Area	% OD	pI	RMM
5	P07724	0.036	1.072	0.469	5.42	66000
	P07724	0.206	1.929	1.380	5.49	65673
	P14733	0.020	0.766	0.366	5.20	65510
	P14211	0.173	2.113	1.107	4.44	59607
	P19226	0.050	1.072	0.614	5.26	58289
	P19226	0.370	3.154	1.414	5.31	58000
10	P19226	0.132	1.501	1.181	5.29	58144
	P14211	0.023	0.949	0.346	4.43	56578
	P27773	0.077	1.317	0.893	5.70	56422
	P27773	0.295	2.603	1.438	5.83	56422
	P09103	0.422	3.246	1.471	4.86	55496
	P09103	0.059	0.551	1.332	4.82	55803
15	P09103	0.114	1.001	1.421	4.88	55496
	P99019	0.096	1.072	1.252	5.72	54585
	P02551	0.050	0.827	0.786	5.11	53541
	P04104	0.043	1.041	0.556	5.54	53246
	P20152	0.026	0.521	0.574	5.12	53099
	P56480	0.054	1.072	0.654	4.96	49835
20	P56480	0.137	1.409	1.316	5.00	49561
	P56480	0.448	3.553	1.472	5.05	49152
	P17182	0.468	3.706	1.542	6.34	46771
	P05784	0.038	1.011	0.497	5.15	46771
	P05784	0.083	1.501	0.737	5.20	46771
	P05784	0.130	1.654	1.056	5.26	46642
25	P29758	0.074	1.072	1.017	5.88	44260
	P99021	0.318	2.695	1.392	5.25	42000
	P99022	0.064	0.827	1.093	5.27	42232
	P99016	0.160	1.715	1.218	6.43	41168
	P35505	0.416	3.093	1.635	6.77	40985
	P14206	0.111	1.562	0.991	4.85	40985
30	P99018	0.016	1.164	0.175	5.27	40622
	Q64374	0.484	4.104	1.367	5.08	33929

	ID	% Vol.	Area	% OD	pI	RMM
5	P50431	0.058	0.521	1.316	6.78	32060
	P16015	1.483	9.769	1.722	6.98	28149
	P16015	0.560	4.165	1.576	6.84	27855
	P16015	0.328	2.909	1.406	6.74	27914
	P00405	0.071	1.409	0.699	4.72	26711
	Q00623	0.020	1.072	0.243	5.45	24664
10	Q00623	0.054	1.592	0.475	5.27	24202
	P14701	0.077	1.746	0.588	4.81	24050
	P02762	0.173	1.776	1.197	4.80	19046
	P02762	0.305	2.542	1.461	4.88	19046
	P99014	0.044	3.553	0.152	4.08	14659
15	P56395	0.236	3.032	1.069	4.88	14567
	P08228	0.468	4.349	1.316	6.14	14537
	P12787	0.224	2.848	1.046	4.91	12433
	P16045	0.173	2.634	0.915	5.06	12381
	P12710	0.255	2.756	1.200	6.33	11946

The positions of some of the DEPs coincide with those of reference spots. Sequencing can be used to check whether they are actually the same.

5 The following protein have been identified as liver markers.

MARKER	GENE NAME	PROTEIN DESCRIPTION	SWISS-PROT AC	METHOD OF IDENTIFICATION
LOM16	CPS1	Carbamoyl-phosphate synthetase I	P07756/P99015	PMF+MS/MS
LOM17	FTHFD	10-Formyltetrahydrofolate dehydrogenase	P28037/P99017	PMF
LOM18	M2GD	Dimethylglycine dehydrogenase	Q63342	PMF
LOMT19	HSP60	Heat shock protein 60	P19226	PMF
LOM20	ERP60	Protein disulfide isomerase ER-60	P27773	PMF
LOMT21	ALDH1	Aldehyde dehydrogenase. mitochondrial	P47738	PMF
LOMT22	SAHH	Adenosylhomocysteinase	P50247	PMF
LOMT23	ALR	Alcohol dehydrogenase	P51635	PMF+MS/MS
LOMT24	GNMT	Glycine N-methyltransferase	P13255	PMF+MS/MS
LOMT25	AOP2	Nonselenium glutathione peroxidase	008709	PMF+MS/MS
LOMT26	-	-	-	-
LOM27	PPIA	Peptidyl-prolyl cis-trans isomerase A	P17742	PMF+MS/MS
LOM28	FABPL	Fatty acid binding protein, liver	P12710	PMF+MS/MS
LOM29	DDT	D-dopachrome tautomerase	035215	PMF+MS/MS
LSEM30	-	Unknown protein	P99032	PMF

25 Referring now to Figures 2-16, these Figures show images of lean controls (left) and ob/ob ("obese") controls (right) relating to one mouse. It should be appreciated that these images cannot fully represent to the eye the differences in expression measurable by computer. Thus, underneath, a bar chart is provided in which the volume of the spot as a percentage of the total volume of all spots is shown on the y-axis. All bar charts relate to 4 mice and resulted from a student T test (p<0.01). For

the Group 1 spots ("OM" designations), only the lean and ob/ob controls are shown. The lean treated and ob/ob treated are omitted, since they did not differ significantly from the respective controls. For the 5 Group 2 spots ("OMT" designations), the ob/ob ("obese") treated bar is also shown. It will be seen to be of similar magnitude to the lean control. A bar for the lean treated is not shown, since the expression did not differ significantly from the lean control. Images for 10 the ob/ob and lean treated were obtained, but are not shown here, in the interests of clarity and simplicity. For the Group 3 spots ("SEM" designation), the four images and bars are shown.

15 **Example 2**

The method of Example 1 was repeated, substituting for 20 the liver tissue 200 micrograms (analytical scale) or 4 mg (preparative scale) of dried gastrocnemius muscle tissue and using the same respective volumes of the protein-solubilising solution as in Example 1. A 2-DGE map was thus obtained, from which DEPs were identified 25 and isolated.

The results are presented in Figures 18-23. Figure 18 30 shows the location of the proteins which has been determined using a set of protein markers as reference spots. Figures 19-23 show the differential expression.

The muscle DEPs were divided into groups as in Example 1, although none fell into Group 3. They were as follows:

Group 1: MOM 31, 32, 33 and 36

Group 2: MOMP 34 and 35

MOM 33 and MOMP 35 were under-expressed in the lean control relative to the ob/ob ("obese") control. The 35 others were relatively over-expressed in the lean

control. Again, bar charts enable the relative magnitude of the spots to be assessed in volume terms.

**Table 2:**

MARKERS	VOL	%VOL	AREA	%OD	OD	pl	Mw
MOM31	0.259	0.075	0.919	1.225	1.515	6.21	118405
MOM32	0.339	0.098	1.347	1.087	1.344	6.25	101518
MOM33	0.072	0.021	0.643	0.459	0.567	5.19	89359
MOMT35	0.095	0.028	1.011	0.43	0.532	5	40803
MOM34	0.231	0.067	1.133	0.954	1.18	6.19	38941
MOM36	0.091	0.026	1.378	0.301	0.372	6.17	32127

The following proteins have now been identified as muscle markers.

MARKER	GENE NAME	PROTEIN DESCRIPTION	SWISS-PROT AC	METHOD OF IDENTIFICATION
MOM31	PCX	Pyruvate carboxylase	Q05920	PMF
MOM32	-	-	-	-
MOM33	-	-	-	-
MOMT34	IRP2	Iron responsive element binding protein 2	Q62751	PMF
MOMT35	-	-	-	-
MOM36	2	-	-	-

**Example 3**

The method of Example 1 was repeated, substituting for the liver tissue 16 mg (analytical scale) or 160 mg (preparative scale) of white adipose tissue and using the same respective volumes of the protein-solubilising solution as in Example 1. A 2-DGE map was thus obtained, from which DEPs were identified and isolated.

The results are presented in Figures 24-49. Figure 24 shows the location of the proteins which has been

determined using a set of protein markers as reference spots. Figures 25-49 show the differential expression.

The white adipose DEPs were divided into groups as in

5 Example 1. They were as follows:

Group 1: WOM 38, 40, 41, 43 - 47 and 51 - 64

Group 2: WOMT 37, 39, 42 and 48 - 50

Group 3: WSEM 65

WOM 39 -41, 43, 46, 47 and 51 were under-expressed in the  
10 lean control relative to the ob/ob ("obese") control.

The others were relatively over-expressed in the lean control. Again, bar charts enable the relative magnitude of the spots to be assessed in volume terms.

Table 3:

MARKERS	VOL	%VOL	AREA	%OD	OD	pl	Mw
WOMT37	0.527	0.088	1.164	0.817	2.292	6.11	122912
WOM38	0.724	0.12	1.654	0.764	2.145	6.97	81427
5 WOMT39	0.161	0.027	0.643	0.563	1.581	6.28	68965
WOM40	0.744	0.124	1.684	0.83	2.328	5.34	67063
WOMT42	0.277	0.046	0.827	0.576	1.615	5.35	62580
WOM41	0.174	0.029	0.735	0.493	1.383	5.29	60937
10 WOM43	0.354	0.059	0.766	0.829	2.326	5.12	53967
WOM46	0.141	0.024	1.011	0.234	0.657	5.15	32756
WOMT48	0.18	0.03	0.827	0.424	1.191	6.16	36889
WOM47	0.329	0.055	1.96	0.3	0.841	5.19	36526
WOMT49	0.364	0.061	0.949	0.749	2.101	6.37	35371
15 WOMT50	9.187	1.529	13.934	1.094	3.069	6.97	32129
WOM51	0.637	0.106	1.439	0.865	2.426	5.35	31989
WOM52	1.644	0.274	2.695	1.037	2.909	6.5	26760
WOM55	0.235	0.039	1.072	0.471	1.322	6.29	22380
20 WSEM65	0.128	0.021	1.746	0.124	0.349	5.39	22172
WOM54	0.195	0.032	1.409	0.269	0.755	5.66	22275
WOM53	0.446	0.074	1.47	0.619	1.736	6.01	22069
WOM57	0.177	0.029	1.133	0.311	0.873	6.5	16454
25 WOM59	0.703	0.117	1.654	0.803	2.252	7.75	14353
WOM60	1.17	0.195	2.328	0.908	2.548	6.6	13775
WOM61	0.607	0.101	1.347	0.814	2.283	7.51	13695
WOM58	0.068	0.011	1.133	0.109	0.305	5.42	13416
WOM62	0.621	0.103	1.868	0.673	1.887	6.28	11151
WOM64	0.484	0.081	1.531	0.556	1.561	6.6	10515
WOM63	0.571	0.095	1.501	0.647	1.816	6.68	10546

30 The following adipose markers have been identified.

MARKER	GENE NAME	PROTEIN DESCRIPTION	SWISS-PROT AC	METHOD OF IDENTIFICATION
WOMT37				
WOM38				
WOMT39				
5	WOM40	GRP75 Mitochondrial stress-70 protein	P38647	PMF+ MS/MS
WOM41				
WOMT42	ALB	Albumin (Fragment)	P07724	PMF+MS/MS
WOM43				
WOM46				
10	WOM47			
WOMT48				
WOMT49				
WOMT50	CA3	Carbonic anhydrase 3 (fragment)	P16015	PMF+MS/MS
WOM51	ANX2	Lipocortin II	P07356	PMF+MS/MS
15	WOM52	CA3 Carbonic Anhydrase 3 (Fragment)	P16015	PMF
WOM53				
WOM54				
WOM55				
WOM57				
20	WOM58			
WOM59				
WOM60				
WOM61	PPIA	Peptidyl-prolyl cis-trans isomerase A	P17742	MS/MS
25	WOM62	Glutathione S-Transferase P 1 (Fragment)	P19157	MS/MS
WOM63				
WOM64	CA2	Carbonic Anhydrase 2 (Fragment)	P00920	PMF+MS/MS
WSEM65				

Example 4

30 The method of Example 1 was repeated, substituting for the liver tissue 400 micrograms (analytical scale) or 4

mg (preparative scale) of brown adipose tissue and using the same respective volumes of the protein-solubilising solution as in Example 1. The weight of sample loaded was 150 micrograms (analytical) or 1.5 mg (preparative).

5 A 2-DGE map was thus obtained, from which DEPs were identified and isolated.

10 The results are presented in Figures 50-62. Figure 50 shows the location of the proteins which has been determined using a set of protein markers as reference spots. Figures 51-62 show the differential expression.

15 The brown adipose DEPs were divided into groups as in Example 1, although there were none in Group 3. They were as follows:

Group 1: BOM 66, 67, 69 - 75 and 77

Group 2: BOMT 68 and 76

Again, bar charts enable the relative magnitude of the spots to be assessed in volume terms.

20 **Table 4:**

MARKERS	VOL	%VOL	AREA	%OD	OD	pl	Mw
BOM66	0.592	0.161	2.082	1.178	1.455	6.44	73811
BOM67	0.043	0.012	0.643	0.329	0.407	5.78	54647
BOMT68	0.047	0.013	1.194	0.18	0.223	5.68	54476
BOM69	0.485	0.132	1.623	1.369	1.691	5.13	49744
BOM71	1.125	0.306	3.277	1.622	2.004	6.64	44857
BOM70	0.635	0.173	2.205	1.246	1.539	6.4	44857
BOM72	0.483	0.131	1.868	1.061	1.311	6.8	38472
BOM73	0.533	0.145	1.654	1.479	1.827	5.18	24316
BOM74	0.048	0.013	1.654	0.123	0.152	4.97	12722
BOM75	0.009	0.002	0.398	0.271	0.335	5.88	12432
BOMT76	6.325	1.722	10.627	2.369	2.927	7.61	12093
BOM77	0.55	0.15	2.787	0.861	1.064	5.14	12111

The following proteins have been identified as brown adipose tissue markers.

	Marker	Gene Name	Protein Description	SWISS-PROT AC	Method of Identification
5	BOM66	ACO2	Aconitate Hydratase	Q99798	MS/MS
	BOM67	-	-	-	-
	BOMT68	-	-	-	-
	BOM69	-	-	-	-
	BOM70	-	-	-	-
10	BOM71	ACO2	Aconitate Hydratase Fragment		
	BOM72	FH	Fumarate Hydratase (Dimer)	P97807	MS/MS
	BOM73	-	-	-	-
	BOM74	-	-	-	-
	BOM75	-	-	-	-
15	BOM76	-	-	-	-
	BOM76	GBP	Lactose-binding Lectin I	P16045	MS/MS

Each of the above-mentioned references is herein incorporated by reference to the extent to which it is relied on herein.

The following claims define some important embodiments of the invention, but should not be construed as detracting from the generality of the inventive concepts hereinbefore set forth.

**APPENDIX 1****A. Assays for compounds that bind to target proteins**

The following assays identify compounds that bind to target proteins, bind to other cellular proteins that interact with a target proteins, and to compounds that interfere with the interaction of the target proteins with other cellular proteins. Such compounds may include other cellular proteins.

The binding compounds may include peptides such as, for

example, soluble peptides, including, but not limited to, Ig-tailed fusion peptides, comprising extracellular portions of target protein transmembrane receptors, and members of random peptide libraries (see, e.g. Lam et

al., 1991, *Nature* 354: 82-84; Houghten et al., 1991, *Nature* 354: 84-86) made of D- and/or L-configuration amino acids, phosphopeptides (including member of random or partially degenerate, directed phosphopeptide

libraries: see, e.g., Songyang et al., 1993, *Cell* 72:

767-778), antibodies (including, but not limited to, polyclonal, monoclonal, humanised, anti-idiotypic, chimeric or single chain antibodies, and FAb, F(ab')<sub>2</sub> and FAb expression library fragments, and epitope-binding fragments thereof) and small organic or inorganic molecules.

Compounds identified via assays such as those described herein may be useful, for example, in elaborating the biological function of the target protein, and for

alleviating insulin resistance disorders. In instances, for example, whereby an insulin resistance disorder situation results from a lower overall level of target protein expression and/or target protein activity in a cell or tissue involved in such an insulin resistance

disorder, compounds that interact with the target protein may include ones which accentuate or amplify the activity of the bound target protein. Such compounds would bring about an effective increase in the level of target protein activity, thus alleviating symptoms. In instances whereby mutations within the target gene cause aberrant target proteins to be made which have an adverse effect that leads to an insulin resistance disorder, compounds that bind target protein may be identified that inhibit the activity of the bound target protein.

The principle of the assays used to identify compounds that bind to the target protein is as described above for the diagnostic assays for DEPs. Thus, it involves reacting the target protein and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a product complex which can be detected. These assays can be conducted in a variety of ways. For example, either the target protein or the test substance can be immobilised onto a solid phase and the target protein/test compound complex anchored on the solid phase detected at the end of the reaction. In one embodiment of such a method, the target protein may be anchored onto a solid surface, and the test compound, which is not anchored, may be labelled, either directly or indirectly.

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected, e.g. using an immobilised antibody specific for target protein or the test compound to anchor any complexes formed in solution, and a labelled antibody specific for the other component of the possible complex to detect anchored complexes.

Where the compound which interacts with the target protein is a cellular protein, any method suitable for detecting protein-protein interactions, especially as described above for the detection of PPs, may be employed  
5 for its identification.

**B. Antibodies for the inhibition of target protein**

Antibodies specific for target protein which interfere with and thus reduce its activity may be used to inhibit  
10 target protein function. Such antibodies may be generated using standard techniques described above, against the proteins themselves or against peptides corresponding to portions of the proteins. These antibodies include polyclonal, monoclonal, Fab fragments, single chain  
15 antibodies, chimeric antibodies, as described above in relation to DEPs.

In instances where the target gene protein is intracellular and whole antibodies are used,  
20 internalising antibodies may be preferred. However, lipofectin or liposomes may be used to deliver the antibody or a fragment of the Fab region, which binds to the target protein epitope into cells. Where fragments of the antibody are used, the smallest inhibitory  
25 fragment, which binds to the target protein's binding domain, is preferred. For example, peptides having an amino acid sequence corresponding to the domain of the variable region of the antibody that binds to the target protein may be used. Such peptides may be synthesised  
30 chemically or produced via recombinant DNA technology using methods well known in the art (e.g. see Creighton, 1983, "Proteins: Structures and Molecular Principles", W.H.Freeman & Co., New York, and Sambrook et al., 1989, "Molecular cloning: A Laboratory Manual", Cold Spring  
35 Harbor, New York.

Alternatively, single chain neutralising antibodies, which bind to intracellular target protein epitopes, may also be administered. Such single chain antibodies may be administered, for example, by expressing nucleotide sequences encoding single-chain antibodies within the target cell populating by using, for example, techniques such as those described in Marasco et al., 1993, Proc. Natl. Acad. Sci. USA, 90: 7889-7893).

In instances where the target protein is extracellular, or is a transmembrane protein, any of the administration techniques described below which are appropriate for peptide administration may be used to effectively administer inhibitory target protein antibodies to their site of action.

**C. Assays for compounds that interfere with target protein/cellular macromolecule interaction**

The target proteins identified by the invention may, *in vivo*, interact with one or more cellular or extracellular macromolecular binding partners, e.g. proteins or nucleic acids. Compounds that disrupt such interactions may be useful in regulating the activity of the target protein, especially mutant target proteins.

The basic principle of the assay systems used to identify compounds that interfere with the interaction between the target protein and its cellular or extracellular binding partner or partners involves preparing a reaction mixture containing the target protein, and the binding partner under conditions and for a time sufficient to allow the two to interact and bind, thus forming a complex. In order to test a compound for inhibitory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound may be initially

included in the reaction mixture, or may be added at a time subsequent to the addition of target protein and its cellular or extracellular binding partner. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the target protein and the cellular or extracellular binding partner is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction of the target protein and the interactive binding partner. Additionally, complex formation within reaction mixtures containing the test compound and a mutant target protein may be assayed. This comparison may be important in those cases wherein it is desirable to identify compounds that disrupt interactions of mutant but not normal target proteins.

The assays can be carried out as described above, *mutatis mutandis*, for other compounds that bind to target proteins, in a heterogeneous or homogeneous format. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the target protein and the binding partners, e.g. by competition, can be identified by conducting the reaction in the presence of the test substance, i.e. by adding the test substance to the reaction mixture prior to or simultaneously with the target protein and interactive cellular or extracellular binding partner. Alternatively, test compounds that disrupt pre-formed complexes, e.g. compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed.

D. Blocking expression of the gene encoding the target protein

Antisense RNA and DNA molecules act to directly block the translation of mRNA by hybridising to targeted mRNA and preventing protein translation. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g. between the -10 and +10 regions of the target gene nucleotide sequence of interest, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalysing the specific cleavage of RNA. (For a review, see Rossi, 1994, Current Biology 4: 469-471). The mechanism of ribozyme action involves sequence specific hybridisation of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. Practical ribozyme molecules are of a DNA counterpart to ribozymal RNA and are preferably of the well known hammerhead type.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the molecule of interest for ribozyme cleavage sites which include one of the following sequences: GUA, GUU and GUC. Once identified, short TNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target protein RNA, containing the cleavage site may be evaluated for predicted structural features, such as secondary structure, that may render the proposed oligonucleotide ribozymal cDNA sequence unsuitable. The suitability of candidate sequences may also be evaluated by testing their accessibility to hybridise with complementary oligonucleotides, using ribonuclease protection assays.

Nucleic acid molecules to be used in triplex helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be 5 designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be 10 pyrimidine-based, which will result in TAT and CGC<sup>+</sup> triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be 15 chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in 20 GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so-called "switchback" nucleic acid molecule. 25 Switchback molecules are synthesised in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

30 Antisense RNA and DNA, ribozyme and triple helix-forming molecules may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. They include techniques for chemically synthesising 35 oligodeoxyribonucleotides and oligoribonucleotides well

known in the art such as, for example, solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors, which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesise antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various well-known modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include the addition of flanking sequences or ribo- or deoxyribonucleotides to the 5' and/ or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

## APPENDIX 2

### Transgenic animals

Additionally, animal models exhibiting insulin resistance disorder-like symptoms may be "engineered" by using, for example, the gene sequence of a DEP or PP in conjunction with well known techniques for producing transgenic animals. For example, gene sequences of target proteins may be introduced into, and over-expressed in, the genome of the animal of interest, or, if endogenous gene sequences of target proteins are present, they may either be over-expressed or, alternatively, may be disrupted in order to under-express or inactivate gene expression of target proteins.

In order to over-express the target gene sequence of a

target protein, the coding portion of the target gene sequence may be ligated to a regulatory sequence, which is capable of driving gene expression in the animal and cell type of interest. Such regulatory regions will be 5 well known to those of skill in the art, and may be used in the absence of undue experimentation.

For under-expression of an endogenous gene sequence of a target protein, such a sequence may be isolated and 10 engineered such that when reintroduced into the genome of the animal of interest, the endogenous gene alleles of the target protein will be inactivated. Preferably, the engineered gene sequence of the target protein is introduced by gene targeting so that the endogenous 15 sequence is disrupted upon integration of the engineered target gene sequence into the animal's genome. Gene targeting is discussed below.

Animals of any species, including, but not limited to, 20 mice, rats, rabbits, guinea pigs, pigs, mini-pigs, goats and non-human primates, e.g. baboons, squirrels, monkeys and chimpanzees may be used to generate insulin resistant disorder animal models.

25 Any technique known in the art may be used to introduce a target gene transgene of a target protein into animals to produce the founder lines of transgenic animals. Such techniques include pronuclear microinjection (Hoppe and Wagner, US Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten *et al.*, 1985, Proc. Natl. Acad. Sci., USA 82: 6148-6152); gene targeting in embryonic stem cells (Thompson *et al.*, 1989, Cell 56: 313-321); electroporation of embryos (Lo, 1983, Mol. Cell Biol. 3: 1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, Cell 57: 717-723). 30 35

For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115: 171-229. The transgenic animals may carry the transgene in all or some only of their cells (see, for example, techniques 5 described by Jakobovits, 1994, Curr. Biol. 4: 761-763). The transgene may be integrated as a single transgene or in concatamers, e.g. head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type 10 by following, for example, the teaching of Lasko et al., 1992, Proc. Natl. Acad. Sci. USA 89: 6232-6236. The regulatory sequences required to such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill 15 in the art.

When it is desired that the target gene transgene be integrated into the chromosomal site of the endogenous target gene, gene targeting is preferred. Briefly, when 20 such a technique is to be used, vectors containing some nucleotide sequences homologous to the gene of the endogenous target protein of interest are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the 25 function of, the nucleotide sequence of the endogenous target gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene of interest in only that cell type, by following, for example, the teaching of Gu et al., 30 1994, Science 265: 103-106). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

35 Once transgenic animals have been generated, the

expression of the recombinant target gene and protein may be assayed using standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyse animal tissues to assay whether 5 integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include Northern blot analysis of tissue samples obtained from the animal, in situ hybridisation analysis, 10 and RT-PCR. Samples of target protein-expressing tissue may also be evaluated immunocytochemically using antibodies specific for the transgene protein of interest.

15 The transgenic animals that express target gene mRNA or elicit target protein transgene peptide (detected immunocytochemically, using antibodies directed against target protein epitopes) at easily detectable levels should then be further evaluated to identify those 20 animals which display characteristic insulin resistant disorder-like symptoms. Such symptoms may include, for example, obesity, glucose intolerance and/or non-insulin dependent diabetes. Additionally, specific cell types within the transgenic animals may be analysed and assayed 25 for cellular phenotypes characteristic of insulin resistant disorders. Such cellular phenotypes may include, for example, abnormal adipocyte differentiation (e.g. abnormal preadipocyte/adipocyte differentiation) and metabolism. Further, such cellular phenotypes may 30 include an assessment of a particular cell types fingerprint pattern of expression and its comparison to known fingerprint expression profiles of the particular cell type in animals exhibiting insulin resistance disorders. Such transgenic animals serve as suitable 35 model systems for insulin resistance disorders.

Once target protein transgenic founder animals are produced (i.e. those animals which express target proteins in cells or tissues of interest and which, preferably, exhibit symptoms of insulin resistance disorders), they may be bred, inbred, outbred or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound target protein transgenics that transgenically express the target protein of interest at higher levels because of the effects of additive expression of each target gene transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the possible need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; breeding animals to different inbred genetic backgrounds so as to examine effects of modifying alleles on expression of the target protein and the development of insulin resistance disorder-like symptoms. One such approach is to cross the target protein transgenic founder animals with a wild type strain to produce an F1 generation that exhibits insulin resistance disorder-like symptoms, such as glucose intolerance, hyperinsulinemia, non-insulin dependent diabetes and obesity. The F1 generation may then be inbred in order to develop a homozygous line, if it is found that homozygous target protein transgenic animals are viable.